The Role of White Adipose Tissue in the Development of Obesity-associated Insulin Resistance and Endogenous Fat Mass Control

Dissertation

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For my dad

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1. Summary

Obesity is linked to a number of comorbidities such as cardiovascular disease, musculoskeletal disease, cancer, and type 2 diabetes. Visceral obesity, i.e. adipose tissue around the greater omentum and mesentery as well as deep subcutaneous layers, is independently and highly correlated to aggravation of several of these diseases. This may be due to inherent properties including higher release of free fatty acids and higher concentrations of several pro-inflammatory cytokines and adipokines which drain into the portal vein promoting the development of (hepatic) insulin resistance and liver steatosis. It was shown that in obese and type 2 diabetic patients, certain inflammatory cytokines are chronically elevated at low levels. Therefore, inflammatory cytokines have been implicated in contributing to or causing insulin resistance in organs like liver, skeletal muscle, and white adipose tissue (WAT) by directly or indirectly interfering with insulin signalling pathways.

The aim of this thesis was first to further elucidate the role of WAT inflammation in the early setting of high-fat diet-induced insulin resistance as well as second, to test the hypothesis that WAT transplantation is capable of interfering with auto-regulatory mechanisms of endogenous WAT mass control.

Regarding the first aim, we were able to demonstrate that a period as short as four days of high-fat feeding lead to hepatic insulin resistance, hepatic steatosis, and increased mRNA expression of the cytokine tumor necrosis factor α. This effect was reversible in animals with an adipocyte-specific deletion of Fas/CD95, a death receptor known to contribute to a pro-inflammatory milieu: despite having a similar degree of hepatic steatosis, expression of pro-inflammatory cytokines was massively reduced in WAT and mice were protected from developing hepatic insulin resistance.

Regarding the second aim, epididymal WAT was transplanted to the mesentery of recipient mice and, thus, drained into the portal vein. Resulting increased total WAT mass was not compensated for by a decrease of endogenous fat mass as could be expected from published observations. In contrast, total endogenous WAT mass was rather increased. We could not allocate this finding to a single mechanism; however, indirect evidence suggests a hyperplastic response of endogenous WAT, i.e. an increase in adipocyte cell number.

Together, these studies provide new insights into the role of adipose tissue to endogenous fat mass control and to the development of obesity-associated insulin resistance.

2. Zusammenfassung

Übergewicht und Fettleibigkeit sind mit den häufigsten Zivilisationskrankeiten wie kardiovaskuläre Krankheiten, muskuloskeletale Krankheiten, Krebs und Diabetes assoziiert. Eine viszerale Fettleibigkeit, d.h. eine Zunahme des weißes Fettgewebes im Bereich des Omentums und des Magen-Darm-Traktes, sowie der tiefen subkutanen Fettschichten im Bauchbereich, ist unabhängig davon mit einem erhöhten Risiko verbunden, eine oder mehrere dieser Krankheiten oder eine schwerere Form davon zu entwickeln. Dies ist auf die unterschiedlichen Eigenschaften der verschiedenen Fettgewebe zurückzuführen. Das viszerale Fettgewebe besitzt eine erhöhte metabolische Rate und schüttet somit mehr freie Fettsäuren sowie mehr pro-inflammatorische Zytokine und Adipokine aus, welche direkt in die Pfortader drainiert werden und so die Leber einem erhöhtem Stress aussetzen. Bei übergewichtigen Menschen und Typ 2 Diabetes Patienten wurde zudem eine chronisch leicht erhöhte Menge von Entzündungsfaktoren im Blut festgestellt. Daher wird angenommen, dass diese Zytokine an der Entwicklung einer Insulinresistenz in Leber, Skelettmuskel oder weißem Fettgewebe beteiligt sind, da sie direkt oder indirekt mit der Insulin-Signalkaskade interferieren können.

Die Ziele der vorliegenden Dissertation waren zum einen den Einfluss der Inflammation in weißem Fettgewebe auf die frühzeitige Entwicklung einer Insulinresistenz zu untersuchen, sowie zum anderen den Effekt einer viszeralen Fett-Transplantation auf die Regulierung der endogenen Fettmasse zu analysieren.

In der ersten Studie konnte aufgezeigt werden, dass eine viertägige hochkalorische Fettdiät ausreicht, eine Leberinsulinresistenz zusammen mit einer hepatischen Steatose und einer Entzündung im weißen Fettgewebe hervorzurufen. Zudem wurde gezeigt, dass eine gezielte Ausschaltung des Fas-Rezeptors (CD95) in Adipozyten, welcher in der pro-inflammatorischen Antwort eine Rolle spielt, die Entwicklung der Leberinsulinresistenz verhindert, ohne das Ausmaß der Lebersteatose zu beeinflussen. Zudem zeigten die Fas-defizienten Mäuse eine massive Reduzierung des inflammatorischen Milieus im weißen Fettgewebe.

In der zweiten Studie wurde epididymales Fettgewebe einer Donor-Maus entnommen und anschließend einem Geschwistertier an den Darm transplantiert (Akzeptor-Maus). Entgegen der zu erwartenden auto-regulatorisch bedingten Abnahme der endogenen Fettmasse als Kompensation des zusätzlich

transplantierten Fettgewebes, welche bei Nagern häufig zu beobachten ist, zeigten Akzeptormäuse eine Zunahme der endogenen Fettgewebsmasse. Der Mechanismus hierfür ist bisher unklar, jedoch deuten indirekte Hinweise darauf hin, dass die Zunahme der Fettmasse auf einer hyperplastischen Antwort, d.h. auf einer Zunahme der Fettzell-Zahl, und nicht auf einer hypertrophen Reaktion, d.h. auf einer Zunahme der Fettzell-Größe, beruht.

Zusammenfassend unterstreichen diese beiden Studien die Wichtigkeit des Fettgewebes in der Entstehung der Obesitas–assoziierten Insulinresistenz und weisen auf eine wichtige auto-regulatorische Rolle des Fettgewebes in der Kontrolle der Fettmasse hin.

3. Abbreviations

ACC	Acetyl-CoA Carboxylase
AFasKO	Adipocyte-specific Fas knock-out
AMPK	Adenosine monophosphate-activated protein kinase
ANCOVA	Analysis of covariance
AS160	Akt substrate of 160 kDa
AT	Adipose tissue
ATGL	Adipose triglyceride lipase
AUC	Area under the curve
BAT	Brown adipose tissue
BM	Body mass
BMI	Body mass index
CEBPα	CCAAT/enhancer-binding protein α
CEBPβ	CCAAT/enhancer-binding protein β
CEBPδ	CCAAT/enhancer-binding protein δ
DAG	Diacylglycerol
DIO	Diet-induced obesity
EE	Energy expenditure
ERK 1 / 2	Extracellular signal-regulated kinases 1 and 2
FA / FFA	Fatty acid / Free fatty acid
FABP	Fatty acid binding protein
FATP	Fatty acid transport protein
FI	Food intake
G-6-P	Glucose-6-Phosphatase
GLUT4	Glucose transporter-4
HFD	High-fat diet
HMW	High molecular weight
HSL	Hormone-sensitive lipase
IKK	Inhibitor of nuclear factor κB kinase
IL	Interleukin
IR	Insulin resistance
IRS	Insulin receptor substrate
JNK	c-Jun-N-terminal kinase

LPL	Lipoprotein lipase
MCP-1	Monocyte chemotactic protein-1
mRNA	messenger RNA
NFκB	Nuclear factor κ B
PEPCK	Phosphoenolpyruvate carboxykinase
PKA/C	Protein kinase A /C
PLIN1	Perilipin
PPARγ	Peroxisome proliferator-activated receptor γ
pTx	portal-transplanted mice
RBP4	Retinol binding protein 4
SOCS3	Suppressor of cytokine signalling 3
SREBP	Sterol regulatory element binding protein
SVF	Stromal vascular fraction
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAG /TG	Triacylglycerol / Triglycerides
TNFα	Tumor necrosis factor α
TLR	Toll-like receptor
UCP-1	Uncoupling protein-1
WAT	White adipose tissue
scWAT	Subcutaneous WAT
mWAT	Mesenteric WAT
rWAT	Retroperitoneal WAT
iWAT	Inguinal WAT
eWAT	Epididymal WAT
WHO	World Health Organisation

4. Introduction

4.1. Obesity

The obesity pandemic has become one of the challenging health problems not only in the Western hemisphere but also in Asia and developing countries. It can be categorised by the crude measure of body mass index (BMI) which is defined as a person's weight in kilograms divided by the square of his height in metres (1). People are considered overweight from a BMI larger than 25 kg/m^2 and obese from a BMI higher than 30 kg/m^2. Overweight and obesity are associated with a number of leading causes of global death, including cardiovascular disease, musculoskeletal disease, cancer, and diabetes.

According to World Health Organisation (WHO) figures from 2008, the number of overweight people with a BMI >25 kg/m^2 has doubled since 1980 affecting now globally more than 20% of the population, i.e. more than 1.4 billion people (Figure 1). Amongst them, more than 1/3 is considered obese. Causes for this increase are named to be inactive sedentary lifestyle, i.e. reduced daily physical activity, a greater than needed daily caloric intake attributable to the availability of more processed energy-dense foods rich in fat and rich in sugar, and a higher degree of urbanization pointing towards less active lifestyle and use of common transport systems. This so called *obesogenic* environment together with genetic predisposition may have led to the sharp increase over the past 30 years or so (2). The constant increase together with the concomitant associated disease development, especially the incident of type 2 diabetes in even younger population, will be one of the future challenges of modern society.

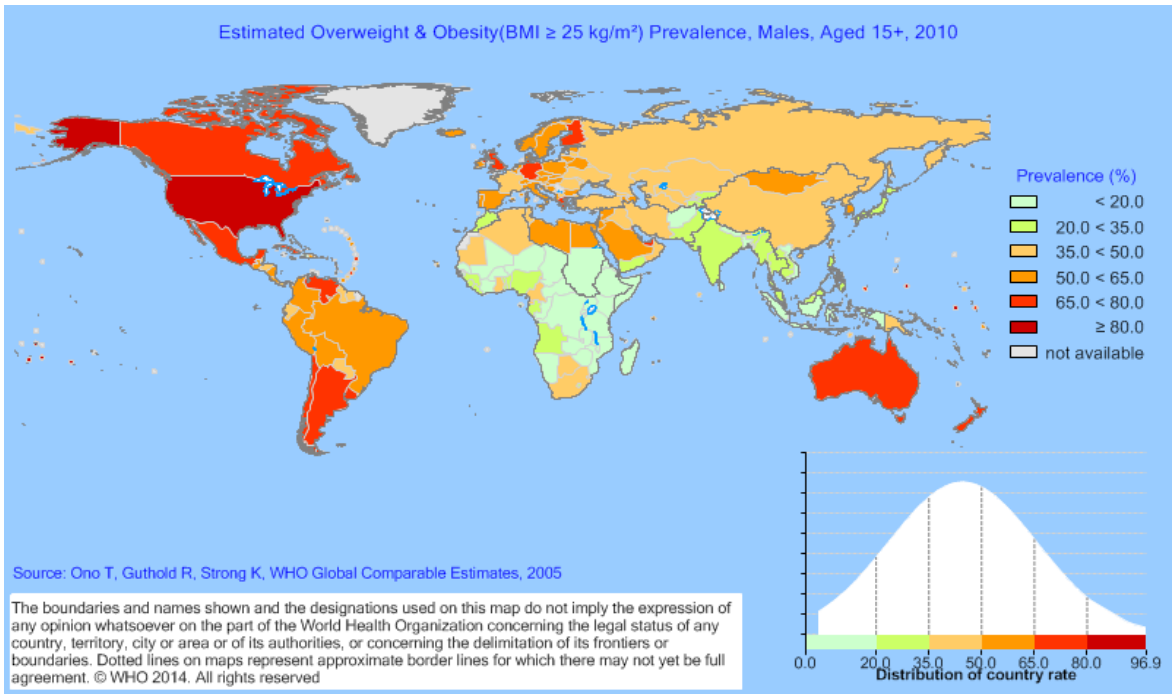

Figure 1. Global map of obesity WHO (3)

4.2. Diabetes

Diabetes mellitus can be subdivided in two different types. Type 1 diabetes mellitus (T1DM), also known as insulin-dependent or juvenile diabetes, is characterised by a malfunction of the pancreas to produce adequate amounts of insulin and hence requires constant administration of exogenous insulin. In contrast, non-insulin dependent or adult-onset diabetes as type 2 diabetes mellitus (T2DM) is described as the inability of the body to make proper use of its insulin, in other words, the development of peripheral insulin resistance. In former times mainly affecting the elderly population, nowadays more and more younger people suffer from T2DM. As it is a silent progressive process, discovery may take up to decades.

According to the WHO global status report from 2010, diabetes is per se the number 5 single cause of global death for non-communicable diseases per year for under the age of 70 years. It affects between 9 and 11 % of the general adult population aged over 25 years dependent on global region and seems to be independent of gross income. If criteria as impaired fasting glycaemia and impaired glucose tolerance are included, proportions are higher than above estimates (4). However, these conditions are intermediate and do not necessarily lead to T2DM.

Approximately 347 million people worldwide suffer from any form of diabetes and more intriguingly, WHO estimates of diabetes-caused deaths are to double up to 2030. This high number almost exceeds even the estimates from a 2004 publication on actual and prospective prevalence of diabetes from 2000 to 2030 (5) which, based solely on demographic changes, was to more than double from roughly 171 million to 366 million people. Hence, action needs to be taken to counteract the development of excessive increase in adipose tissue mass in people.

However, not just simply reducing adiposity but rather understanding its underlying genetic and environmental influences (see e.g. 6 for recent review) and the complex body's system of regulating its need for foods, the interplay of satiety signals, and ultimately increase or decrease of body and fat mass is the major challenge of medical research (7), as e.g. dieting and effective weight loss for overweight or obese people is ineffective over the long term (e.g. 8). Additionally, sole focus on body mass by BMI does not seem to represent the full picture of possible disease status and other indices have been used like waist circumference and waist-to-hip ratio, which on the one hand are easily measurable but on the other hand have some drawbacks as well (see e.g. 9; 10). More recently, several reports have again lead to scientific discussions about usefulness of categorising people with BMI > 25 kg/m^2 as overweight and >30 kg/m^2 as obese together with associations of increased risk for diseases (e.g. 11; 12-14). The interested reader is referred to a recent perspective comment by Ahima and Lazar (15). This may highlight the importance of understanding not only proper function of adipose tissue but also its distribution sites in the body which show site-specific particularities (16) together with proportions of adequate muscle mass simply pointing to the complexity of the system as a whole being difficult to categorise with a simple height-by-weight formula.

4.3. Adipose tissue

Adipose tissue (AT) can be largely subdivided histologically and functionally into white and brown. White adipose tissue (WAT) consists to great extent of large lipid-droplet containing adipocytes, adipocyte precursors, and other cells types commonly called stromal-vascular fraction (SVF) which will be further described in detail below. In contrast, brown adipose tissue (BAT) and its adipocytes are characterised by their colour due to the high density of mitochondria and, compared

to WAT, contain multiple lipid droplets. The main function is not to store excessive energy but rather to produce heat by non-shivering thermogenesis mediated by uncoupling protein-1 (UCP-1) in the respiratory chain. More recently, a third type called ‘beige’ or ‘brite’ adipocyte has been identified residing mainly in WAT and probably having the same progenitor cell being able, dependent on stimuli, to differentiate into either white or beige adipocyte (see also 17). However, beige AT and BAT will not be considered to great extent in this introduction and the interested reader is referred to recent publications and reviews about its existence and function in neonate and adult human beings (e.g. 18; 19-23).

4.4. Development of WAT

The development of adipose tissue starts, dependent on species, at early stages of fetal development but seems to be site-specific with time differences for different depots as well. For example, in human, pigs and also some rodent but not bovine species, subcutaneous WAT (scWAT) apparently seems to develop prior to intra-abdominal, e.g. mesenteric WAT (mWAT) or retroperitoneal WAT (rWAT) (24-31). Remarkable even at that time point is the close association between developing adipocytes and vascularisation which distinguishes depots already at such early stage (32). It is important to note that in both prenatal and perinatal periods, nutritional and hormonal influences seem to imprint subsequent development of adipose tissue. For an overview, see Poulous et al. (31). Hence, in utero programming of AT as determinant of later (mal-) developments to the disadvantage of an unhealthy adipose phenotype has to be considered as well, although data on human are sparse and, hence, knowledge stems mostly from large animal studies with sheep and pig.

It should be noted that there is considerable turnover of adipocytes, i.e. programmed cell death and adipogenesis at substantial rates of approximately 10% per year throughout adult human life span and it was estimated that overall adipocyte half-life is around 8.3 years (33).

Much research has been devoted to understand the development/differentiation of pre-adipocytes into adipocytes. A coordinated transcriptional cascade is responsible for proper adipocyte differentiation until a mature adipocyte reaches its final stage having three primary characteristics

including proper lipid storage capacity, insulin sensitivity, and endocrine properties (34; 35). At the centre of this network are two master regulators, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (CEBPα) which are indispensable for correct adipocyte differentiation and insulin sensitivity, respectively (36; 37). Well before these two factors, others like CCAAT/enhancer-binding protein β (CEBPβ) and δ (CEBPδ) act in concert at the stage of determining the fate of precursor cells towards adipocyte development and induce the expression of PPARγ. Ablation of *pparγ* in embryonic stem cells leads to embryonic lethality (38). Further research by Rosen et al. (39) and Koutnikova et al. (40) showed that *pparγ* knockdown lead to severe lipodystrophy. *pparγ* has two isoform, *pparγ1* and *pparγ2* giving rise to the corresponding PPARγ1 and PPARγ2 polypeptide, respectively. While PPARγ1 is expressed in many tissues, PPARγ2 is restricted to adipose tissue. Interestingly, knockout of adipose-*pparg2* can be compensated in part by PPARγ1 (41). Similarly, CEBPα knockout in mice comes with early lethality due to the inability of these animals to produce glucose as CEBPα is indispensable for gluconeogenesis in liver (42). When being knocked out in all tissues apart from liver, it was demonstrated that CEBPα is required for formation of WAT, but not necessarily BAT (43). Additionally, it was suggested that CEBPα works in concert with PPARγ being responsible for proper insulin function in the mature adipocyte (43; 44). It was shown in cell models that CEBPβ and CEBPδ are expressed earlier than CEBPα in adipogenesis and that they are responsible for CEBPα expression (45; 46). These findings were extended to the ability for CEBPβ to induce expression of PPARγ2 (47). However, when CEBPβ and CEBPδ were knocked out simultaneously, neonatal mice had a defect in producing adipose tissue but there was some residual expression of PPARγ and CEBPα suggesting some redundancy in the system and an additional role downstream of PPARγ and CEBPα since absence of both isoforms prevents terminal adipogenesis (36; 37). A simplified model of the main factors involved can be seen in Figure 2. It should be noted that several other factors like activating protein-1, signal transducers and activators of transcription, Krüppel-like factors, sterol regulatory element binding proteins (SREBPs), and wingless type MMTV integration site family members (Wnts), to name the most prominent ones, are involved as well in positively and/or negatively regulating each of these factors at different stages. Besides these transcription

factors, adipogenesis can also be hormonally controlled by thyroid hormones, steroid hormones, peptide hormones and also glycoproteins (reviewed in 48).

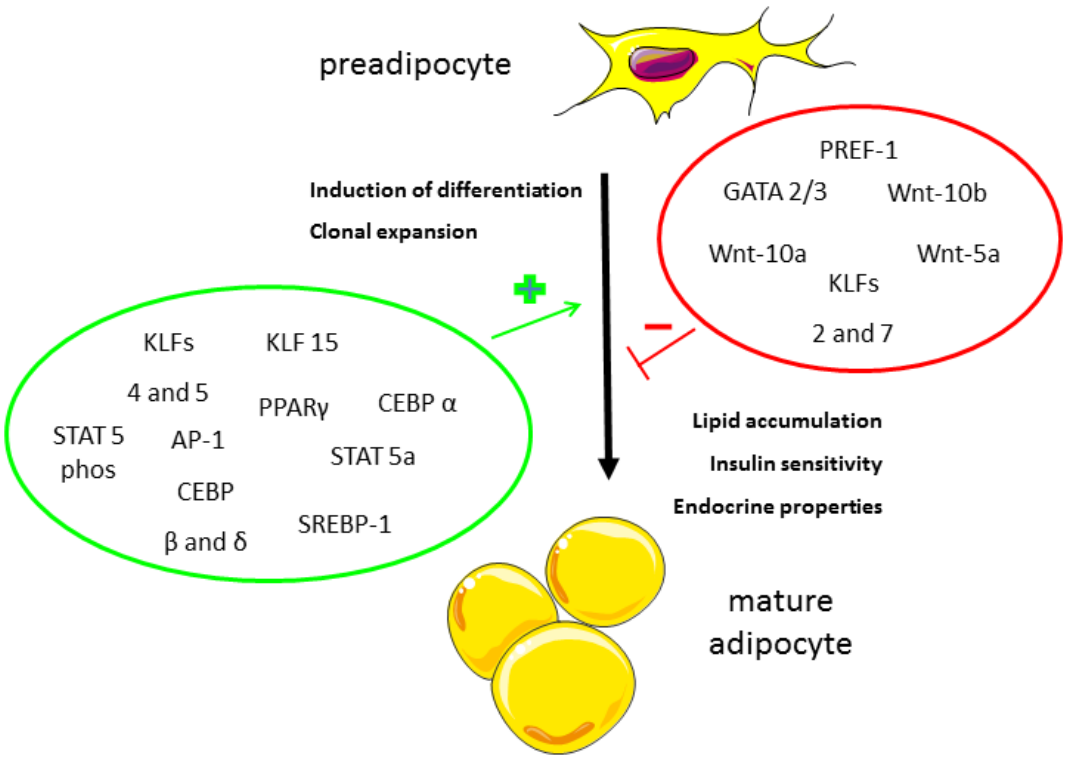

Figure 2. (created with Servier Medical Art www.servier.com). There is no timed order of factors positively (+) or negatively (-) regulating adipogenesis. AP-1 = group of activating protein-1, CEBP = CCAAT/enhancer-binding protein, STAT = signal transducers and activators of transcription, KLF = Krüppel-like factors, PPARγ = peroxisome proliferator-activated receptor γ, SREBP-1 = sterol regulatory element binding protein 1, GATA = (A/T)GATA(A/G) binding protein, PREF-1/DLK1 = delta-like 1 homolog (Drosophila)

4.5. Composition of WAT

Besides mature adipocytes and immature pre-adipocytes, adipose tissue comprises of a number of other cell types including endothelial cells, pericytes, fibroblasts, several immune cell types like macrophages, eosinophils, natural killer cells, B-cells, T-cells, to name just a few, neuronal cells, and others (49-52).

How the composition thereof may change during development of obesity can be exemplified seen in Figure 3 when mice were challenged with a high-fat diet (HFD) for 8-12 weeks to promote obesity and their total number of leukocytes of the SVF was analysed for its composition. Hence, although changes in composition may seem to be of minor extent, underlying physiological output will be altered to great extent as will be described later.

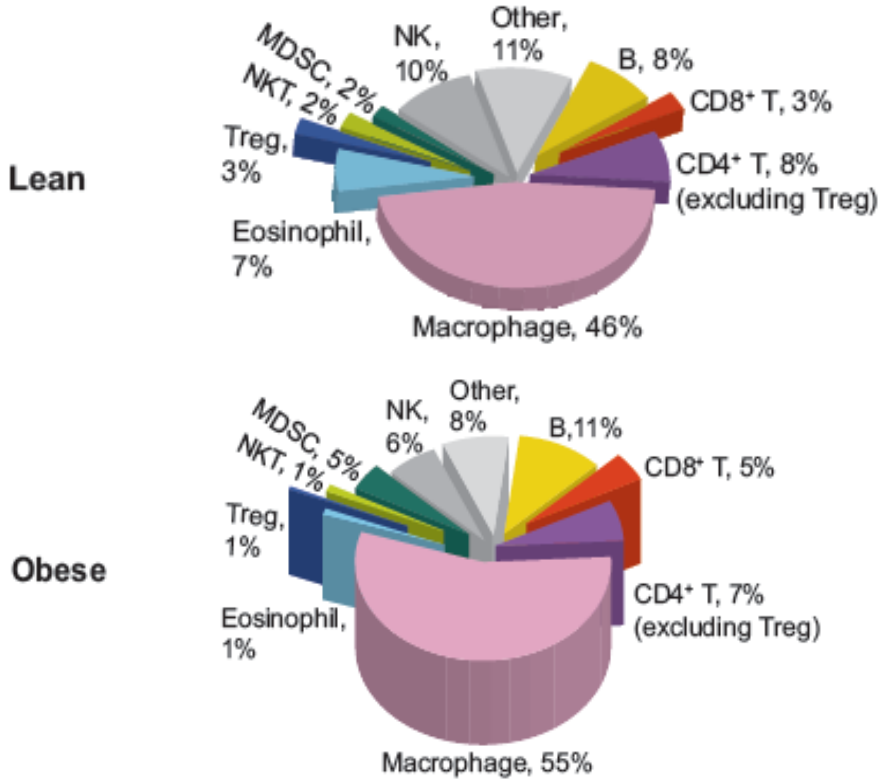

Figure 3. (from (53)) Treg = regulatory T cells, NKT = natural killer T-cells, MDSC = myeloid derived suppressor cells, NK = natural killer cells, B = B-cells, T = T-cells

Physiologically, this was shown to be important for the development and maintenance of insulin resistance in HFD animal models. In 2003, two groups could demonstrate that infiltration of macrophages was causally linked to insulin resistance (IR) as these macrophages secrete large amounts of pro-inflammatory cytokines which interfere with normal functioning of WAT (49; 50). Weisberg et al. (49) could show that total number of macrophages increased from 5-10% in lean individuals to up to 50% of total cells in obese subjects, hence not only qualitative composition as shown above but rather quantitative changes towards a pro-inflammatory milieu was demonstrated to be causally linked to IR.

More recently, several papers also proved that either phenotypic changes of resident AT macrophages or infiltration of other immune cell types like T-reg cells, natural killer T-cells, $CD4^+$ and $CD8^+$ cells may be involved even at earlier time points to either induce negative commitment to insulin resistance (IR) and dysfunction or contribute in concert with other factors to this deleterious development (52; 54-58). Further research is clearly warranted to deduce timely cascade of invasion/attraction

of non-resident cells into WAT and their underlying function both in healthy expansion of WAT and disease development.

4.6. Site-specific differences of WAT

Adding to the complexity, location of WAT seems to be "*a*" if not "*the*" crucial point in determining its function, reacting to *obesogenic* environment, and finally being determinant for health status. Simply seen, storage sites can be divided into subcutaneous, i.e. between dermis and muscle, and visceral storage, i.e. all depots in the intra-abdominal cavity including mWAT, rWAT and omental WAT in human beings. Additionally, centrally-located depots (i.e. omental and mesenteric and also deep-subcutaneous central WAT) are more closely related to deleterious outcomes then peripheral depots (i.e. subcutaneous hip and gluteal-femoral WAT) (59; 60). A closer look defines the drainage sites of these depots as well, i.e. if the factors released by WAT are directed into systemic circulation (scWAT, rWAT) or into portal circulation (mWAT, omental WAT) exposing in a first passage the liver (9; 60-64). In rodent models, an additional depot around the gonads – commonly called perigonadal WAT for both sexes and/or epididymal WAT, eWAT, for male mice – located inside the intra-abdominal cavity but draining into the systemic circulation has to be considered as it is one of the most reacting sites during an *obesogenic* challenge (62-65) and is frequently used for WAT analysis.

The site of adipose is of importance as different depots possess different properties in storing excess energy, reacting to diverse stimuli and releasing factors called "adipokines" into circulation. As mentioned in the beginning, not merely total adipose tissue mass but rather its distribution may be more closely linked to deleterious outcomes, foremost associated to the metabolic syndrome.

Adipocyte size in humans, for example, is larger in mesenteric and perirenal depots and smallest in omental depots whereas scWAT shows intermediate size (31; 60). Additionally, scWAT is more heterogeneous and contains both unilocular adipocytes together with smaller multilocular adipocytes whereas visceral fat is more uniform (63). This may have implications as adipocyte turnover may occur at different rates in these depots and the capacity to recruit more adipocytes (hyperplasia) is associated with healthy expansion of adipose tissue in general (60; 63; 66).

In rodents, however, eWAT has larger adipocytes than scWAT or mWAT and responds more hypertrophic under *obesogenic* HFD challenge (e.g. 67). Nevertheless, mWAT has much higher lipolytic response to e.g. catecholamines, i.e. the capacity to break down and release free fatty acids (FFA) into circulation in situation when energy is needed (68). This poses an extra threat to excessive large visceral depots as FFAs released in great excess have deleterious effects on other organs (69). Conversely, the anti-lipolytic effect of insulin is lower in mWAT versus scWAT (60; 70; 71). In rodents, the situation of intra-abdominal WAT is also site-specific. For example Wueest et al. (67; 72) could show that isolated eWAT, mWAT, and scWAT have different basal lipolytic rates, different inhibition rate under insulin challenge and that these inherent properties are differentially altered under HFD challenge.

Importantly, adipocytes retain their characteristics when isolated and cultured (60; 73) and it seems that even when autologously transplanted, keep their properties and original function intact. However, this was not uniformly observed (65; 74). In these settings, transplanted donor tissue and proper re-vascularisation as well as drainage site may be a decisive factor for different outcomes (75; 76).

More recent reports provide therefore support for intrinsic distinctions in adipocyte precursor cells from several depots in rodents, a divergent capacity to differentiate and substantial differences in gene expression influencing adipogenesis and capacity of releasing different factors (77; 78). Lately, large-scale gene expression analysis and secretome analysis of various human and rodent adipose tissue depots has provided conclusive insights into the differences between depots, their alterations in obesity and under HFD, and the complexities when comparing “adipose tissue” with each other (79-87).

4.7. Adipose tissue as secretory organ

Besides its function as a cushion and storage site for energy when available in excess and its release when in demand, WAT’s role in releasing autocrine, paracrine and endocrine factors has become evident during the past 20 years.

The main function of adipocytes is to take up fatty acids (FA), store them properly as triacylglycerols (TAGs), or commonly named triglycerides (TGs), and release them in situations of energy need. For that purpose, TGs are transported in

the bloodstream via lipoproteins (chylomicrons, very-low density lipoproteins) and are unloaded in the presence of the endothelial-bound lipoprotein lipase (LPL) of the target cell (88). Subsequently, the released FAs are taken up by membrane transporters like fatty acid translocase (FAT/CD36), fatty acid transport proteins (FATPs), plasma-membrane bound FATP (FATPpm), and long-chain fatty acid Acyl-coenzyme A synthetase (ACSL), (89-91). They then are intracellularly shuttled by fatty acid binding proteins (FABPs) to the correct site in the cytoplasm and re-esterified to TAG (92). The other way round, several lipases are necessary for breaking down TAG to FAs so that they can be made of use for energy production or serve as signalling events. Foremost, upon lipolytic stimuli and activation of protein kinase A (PKA), perilipin (PLIN1), a lipid-coating and protecting protein, is phosphorylated which promotes the release of comparative gene identification-58 (CGI-58), which subsequently recruits hormone sensitive lipase (HSL) to the corresponding lipid droplet. CGI-58 binds with adipose tissue triglyceride lipase (ATGL) and both translocate to the droplet where ATGL catalyzes the first step from TAG to diacylglycerol (DAG) releasing one FA. PKA also phosphorylates HSL which then attaches to the lipid droplet surface and catalyzes the second step from DAG to monoacylglycerol (MAG) yielding another FA. Finally, monoglycerol lipase is responsible for the last hydrolyzation step yielding glycerol and another FA (93-99). Figures 4 and 5 illustrate current views on above described pathways.

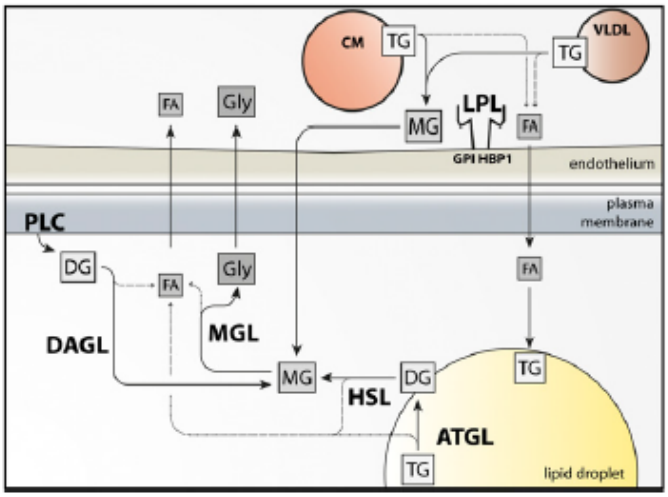

Figure 4. (from (88)) MG = Monoacylglycerol, DG = Diacylglycerol, TG = Triacylglycerol, FA = Fatty acid, PLC = Phospholipase C, DAGL = Diacylgylcerol Lipase, LPL = Lipoprotein Lipase, GP1HBP1 =

glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1, Gly = Glycerol, CM = Chylomicron, VLDL = very low density lipoprotein

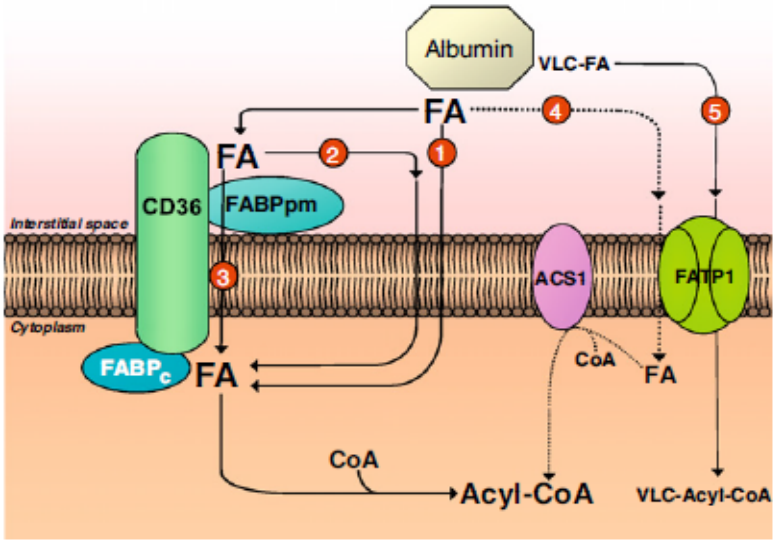

Figure 5. (Reprinted from (100) with permission from Elsevier) FA = Fatty acid, ACS1 = AcylCoASynthetase 1, FATP1 = Fatty acid transport protein 1, FABP = Fatty acid binding protein (c = cytosolic, pm = plasma membrane-bound), CoA = Coenzyme A, VLC-FA = very long chain fatty acid

Importantly, both the intermediate products of TAG catabolism and the released free FA exert numerous signalling functions and e.g. intervene directly with other kinases, as well as regulate a number of transcription factors besides their role as energetic source (93; 101; 102).

4.8. Adipokines

During the past two decades, a second and equally important function of WAT has been described, namely the production and release of autocrine, paracrine, and endocrine acting molecules named adipokines and also cytokines from adipocytes per se or from cells of the SVF compartment of WAT. The origin of the most important and best characterised ones and their function in metabolism and related to WAT changes will be described briefly in the section below. In general, this has made WAT to one of the key organs in regulating both its own metabolism and influencing other organs like skeletal muscle, liver, brain, pancreas, and surrounding vasculature in diverse processes as lipid and carbohydrate metabolism, state of inflammation, blood pressure, energy expenditure and feeding status (31; 103).

Leptin has been one of the first adipokines to be discovered. It is the product of the *ob* gene (104), mainly produced in adipocytes and in low levels in several other organs (105; 106). Leptin is released into the bloodstream, increases with weight gain, decreases during weight loss and fasting and is a direct signal of fat store. Large adipocytes release more leptin than small ones, subcutaneous WAT more than omental one (107). Leptin, together with insulin, acts centrally to directly inhibit appetite (108), and low levels of leptin inhibit action of anabolic state, i.e. growth hormone, thyroid hormone, and sexual hormone release and lowers thermogenesis (109-111). Its production is regulated by several factors including insulin, inflammatory cytokines, glucocorticoids as well as also by meal ingestion or fasting (112; 113).

Leptin acts through the leptin receptor which is the product of the *db* gene, (114) being highly expressed in certain brain regions, especially the hypothalamus, and, hence, central action after crossing the blood-brain-barrier makes leptin to act as a direct satiety signal and fat mass scale. Additionally, leptin acts peripherally on liver, skeletal muscle and adipose tissue itself again as these tissues express one or several isoforms of the leptin receptor (115; 116). Direct actions of leptin in the brain involve inhibition of appetite, stimulating processes like thermogenesis, fatty acid oxidation, and reducing body weight (106; 117). In the periphery, leptin e.g. induces lipolysis in adipocytes and skeletal muscle as well as increases lipid oxidation in the liver (118-121). Additionally, mounting evidence suggests a direct or indirect role of leptin in maintaining glucose homeostasis, especially in peripheral insulin sensitivity (108). This made leptin an ideal candidate for therapeutic use in obesity. However, in obesity, high levels of circulating leptin are observed even when corrected for adipose tissue mass which translates to a relative leptin resistance or insensitivity in obesity state (122). This resistance is immanent both centrally and in the periphery (123; 124).

Adiponectin has also been discovered in the mid-1990s (125) as direct secretory product of adipocytes, and, conversely to leptin, circulating adiponectin was shown to be decreased with increasing fat mass in diverse populations and diet-induced obesity (DIO) models (126-128). Moreover, it is inversely related to risk development of obesity, IR, T2DM, and cardiovascular disease (129; 130).

Adiponectin acts via adiponectin receptors 1 and 2 (ADIPOR1 and 2, respectively) which are ubiquitously expressed and may exert some redundancy when knocked-out selectively (131). Although adiponectin is fairly stable in circulation, its plasma half-life is only 45-75 min which suggests rapid clearance by other organs of which the liver is the main one (132).

Adiponectin secretion in humans is higher from omental than scWAT depots and exists in several multimeric complexes generally termed low-molecular weight, LMW, or high-molecular weight, HMW, adiponectin of which only the latter shows bioactivity on receptors (133). It should be noted that the decrease of circulating adiponectin seen in T2DM is mostly seen in the HMW form and focusing on this form even strengthens above mentioned correlations (134). It was shown to promote pancreatic beta cell function, i.e. probably increase insulin secretion (135), and survival by activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and also Akt (135; 136). Additionally, adiponectin exerts cardioprotective function probably via reducing ceramide levels (137). Adiponectin's action on skeletal muscle improving peripheral insulin sensitivity via increasing glucose uptake seems to be mediated by adenosine monophosphate-activated protein kinase (AMPK) activation (138; 139), however doubts remain as to whether the HMW multimeric form, which is the predominant plasma circulating adiponectin, exerts the same role as most early studies demonstrating an effect in muscle used globular adiponectin (summarised in 140). On the contrary, adiponectin suppresses hepatic glucose output via priming hepatocytes to the effects of insulin. It reduces gene expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-P), two key regulators of gluconeogenesis (141). This effect on liver seems to be both AMPK-dependent and independent. Concerning adipose tissue, adiponectin seems to exert autocrine and paracrine effects. Overexpressing adiponectin leads to healthy expansion of WAT, i.e. an increase in adipocyte number (hyperplasia) rather than hypertrophy of existing adipocytes in a genetic mouse model of obesity together with a lower degree of inflammation (142; 143). Furthermore, adiponectin has profound anti-inflammatory properties as it suppresses growth and proliferation of macrophage progenitors (144) and reduces release of pro-inflammatory cytokines when co-incubated with human macrophages (144) whereas release of anti-inflammatory interleukin-10 (IL-10) is increased (145; 146). Importantly, these anti-inflammatory

properties are not limited to WAT but rather seem to be globally as several macrophages residing at different organs are positively influenced.

4.9. Further adipokines

Besides these two well-characterised adipokines, several others have been found and are still to be discovered. Some of them are exclusively expressed in adipocytes, some also in other cell types but predominantly in adipocytes. Amongst them are those without hormonal function like apolipoprotein A which is a component of TG-rich lipoproteins, transforming growth factor beta which regulates preadipocyte proliferation and differentiation, and also LPL whose role is described above. Other factors exerting local and more systemic functions include Adipsin which stimulates TG storage; Apelin having an effect on cardiovascular function and fluid retention; acylation-stimulation protein being involved in TG synthesis; omentin which may be involved in insulin action; serum retinol binding protein 4 (RBP4) which increases with obesity and promotes IR (147). Similarly to RBP4 is resistin, at least in rodents, whereas its role in humans is less clear as there is some controversy about its origination, preadipocytes, adipocytes (148) or mononuclear blood cells (149; 150); and visfatin which was initially supposed to be an insulin mimetic being higher expressed in visceral vs. scWAT, however, more recent data demonstrated that it is an extracellular form of nicotinamide phosphorybosyl transferase being essential in NAD biosynthesis (151; see 152 for an overview). More recently, adipocyte fatty acid binding protein (AFABP or FABP4), an intracellular fatty acid transporter, has been implicated in IR and dyslipidemia as it was shown to be elevated in development of T2DM and the metabolic syndrome (153; 154). An overview about other adipokines can be found in Catalan et al. (155) or Maury and Brichard (156).

4.10. Inflammation and inflammatory cytokines in WAT

As indicated above, WAT contributes to inflammatory state in health and disease state. Adipocytes, but mostly other cell types from the SVF secrete both pro-inflammatory cytokines like tumour necrosis factor alpha (TNFα) and interleukin-6 (IL-6) or interleukin-1 (IL-1) as well as anti-inflammatory cytokines like IL-10 and

chemokines like monocyte chemotactic protein-1 (MCP-1) which modulate local and systemic processes. Obesity is associated with chronic low grade inflammation, i.e. elevated circulating pro-inflammatory cytokines, which to a large extent stem from WAT (157; 158). In this regard, macrophages, either resident or infiltrating, play an important role. They show roughly two phenotypes: classically pro-inflammatory M1 macrophages and alternatively-activated anti-inflammatory M2 macrophages (159). However, distinction may somewhat differ in a broad continuum. Nevertheless, obesity is associated with a huge increase in total macrophage number (indicated above) and additionally, a switch from M2 to M1 macrophages (159). M1 macrophages are usually recruited to sites of lesion or damage and secrete pro-inflammatory cytokines like IL-6 and TNFα, whereas M2 macrophages release for example IL-10. These factors have direct, local effects on adipocytes, as well as peripheral effects by interfering with insulin signalling pathways in liver and skeletal muscle. The complex interplay between residing and or invading cell types during developmental changes of WAT in obesity was recently extensively reviewed (53; 160-163) and is summarized in descriptive ways in figure 6 which displays the immune cell populations in WAT and figure 7 which shows interactions between these cell types and macrophage polarization in development of obesity.

Two of the most prominent pro-inflammatory cytokines released by WAT and being somehow involved in the stages from obesity to IR will be further described below.

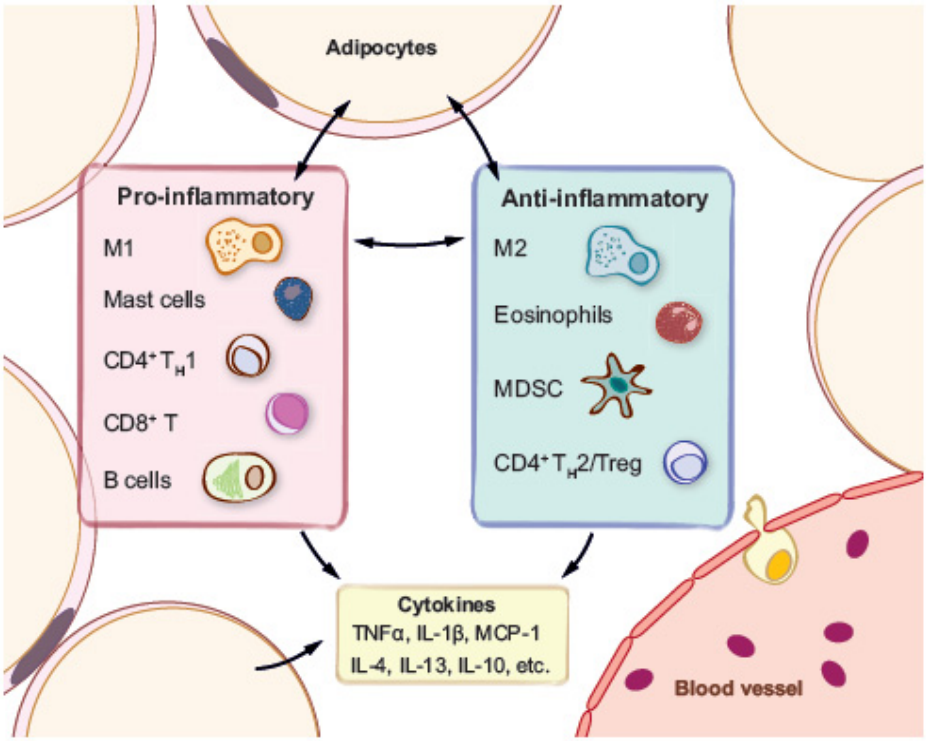

Figure 6. (from (53)) For abbreviations, see text.

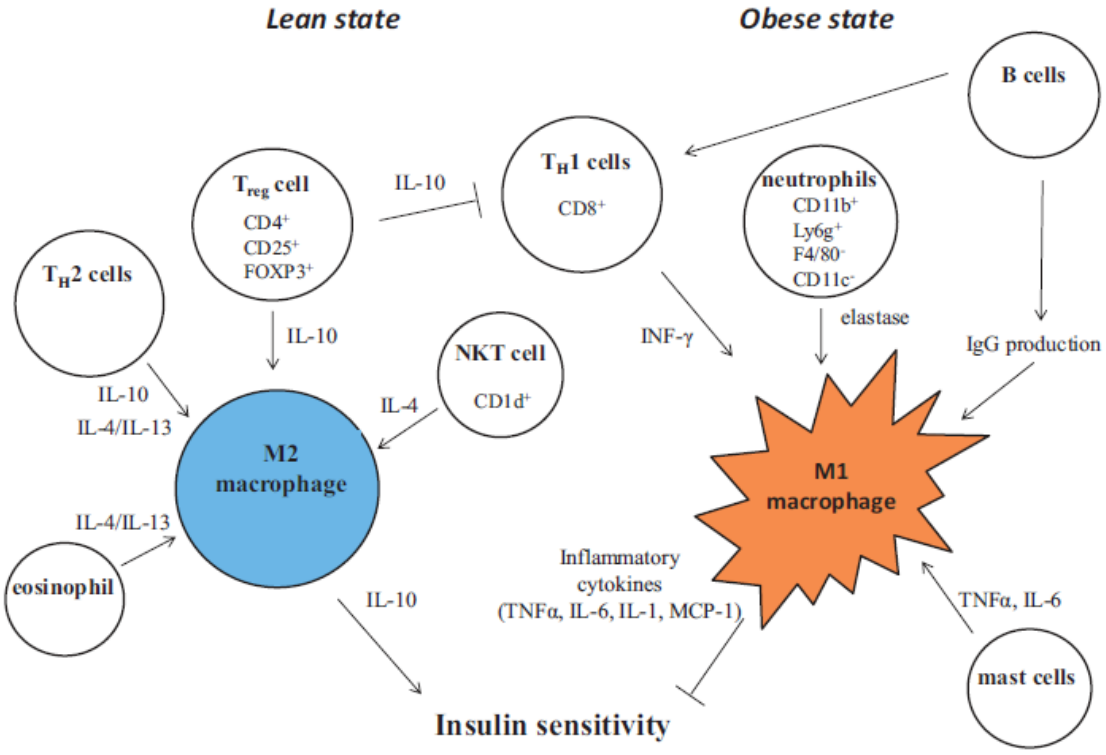

Figure 7. (from (161)) IL = interleukin, IgG = Immunglobulin G. For other abbreviations, see text.

TNFα can be synthesized by adipocytes but the main contribution in obese state stems from M1 polarized macrophages (49). It was the first cytokine derived from WAT which provided a link between obesity, inflammation, and development of T2DM (164). It is released into circulation and can exert both local paracrine effects as well as endocrine effects on diverse target cells and tissues (e.g. 165). Direct effects on adipocytes are reported to be inhibition of lipogenesis, an increase in lipolysis, and a decrease in glucose transporter-4 (GLUT4), CEBPα, PPARγ, Perilipin, fat-specific protein 27 and adiponectin expression amongst others (166; 167). TNFα expression and release is increased in obesity (168) and can directly impair insulin signalling in WAT, muscle, and liver (169-172) whereas deletion of TNFα or its receptors protects animals under *obesogenic* HFD from deterioration of insulin sensitivity and glucose tolerance (164; 173). Concerning impaired insulin signalling, TNFα acts on the insulin receptor substrate (IRS) phosphorylation sites through c-Jun-N-terminal kinase (JNK) (174) and inhibitor of nuclear factor κB (NFκB) kinase (IKK) and through activating suppressor of cytokine signalling 3 (SOCS3) (175). Thereby, it can furthermore enhance other pro-inflammatory cytokines like IL-6 or IL-1 (176). Additionally, it inhibits fatty acid oxidation through protein phosphatase 2C and this further leads to an accumulation of DAGs which may further impair IRS signalling (177). Thus, it seemed promising to use neutralizing antibodies to reduce circulating TNFα. Whereas this strategy is successful in rodents (e.g. 54; 164), results in humans are not as clear (178; 179) and warrant further investigation in larger settings.

Being discovered in the mid-1980s (180), IL-6 gained widespread considerable and controversial interest through its diverse properties in a short- and long-term setting (181-186; summarised in 187) as it is associated with a number of diseases like rheumatoid arthritis, Crohn's disease or some form of cancers (188). IL-6 is expressed and secreted by a large number of cell types including adipocytes, preadipocytes, macrophages, and endothelial cells amongst others (e.g. 60; 189) and released into circulation whereas WAT is supposed to contribute around 1/3 (190). Like many other cytokines and adipokines, site-specific differences in expression and secretion exist (9) with omental WAT releasing more IL-6 than scWAT. Its expression and release into circulation in obesity and T2DM is elevated similar to TNFα (191; 192) and it may directly impair insulin signalling either via

downregulating IRS (193) or upregulating SOCS3 in several insulin-targeting cell types (194-196). As it has lipolytic function as well (197), it may further promote release of FFA into circulation which, as mentioned before, may also interfere with insulin signalling (191). Furthermore, it suppresses adiponectin production (198) and also LPL activity (199). IL-6 acts either via IL-6 receptor or the pleiotropic glycoprotein 130 (gp130) receptor on its target cell (200; 201), which adds to the complexity of this cytokine. Knocking IL-6 out on a whole-body level leads apparently to a complex phenotype with in part opposing results (202; 203). However, a more recent study confirmed that these animals develop mature onset obesity when fed a normal chow diet and develop IR, liver steatosis and inflammation suggesting some necessary roles beyond its promoting pro-inflammatory role (204) and hence targeting its receptors may be more promising (188). However, a certain amount of IL-6 signalling also seems to be indispensable as for example IL-6R alpha deficiency specifically in hepatocytes leads to insulin resistance and glucose intolerance (205).

4.11. Insulin Signalling and Resistance

Insulin is an anabolic hormone produced in the islets of Langerhans in the pancreas and is released upon a rise in blood glucose with its main function to keep blood glucose concentration in a narrow range. For that purpose, it stimulates glucose uptake into target tissues like skeletal muscle and WAT. In liver and muscle, it promotes glucose storage into glycogen and inhibits glycogen breakdown. In addition, insulin suppresses hepatic gluconeogenesis which maintains glucose concentration during fasting. Only a minor portion of the circulating glucose is taken up by WAT pointing to an important function of skeletal muscle for glucose clearance. The glucose taken up by WAT is mainly used as backbone for TAG formation by providing intracellular substrate for glycerol-3-phosphate (206). Furthermore, insulin promotes uptake of circulating TAG and FAs via LPL activity and FATP1 translocation to the plasma membrane and synthesis of FAs via acetyl-CoA carboxylase (ACC) in adipocytes as well as intracellular TAG formation. It suppresses FA release into circulation contributing to its postprandial clearance via reducing cyclic adenosine monophosphate (cAMP) levels and PKA activity, as well as indirectly inhibiting ATGL messenger RNA (mRNA) levels and upregulating fat-specific protein 27 (207).

Insulin acts via binding to the insulin receptor, a tyrosine kinase, which leads to initiation of a signalling cascade via phosphorylation of IRS. Subsequently, further downstream signalling cascades like for example in skeletal muscle, liver, and WAT the phosphatidyl inositol 3-kinase (PI-3-K) - phosphatidyl inositol-dependent kinase (PDK) - serine/threonine protein kinase Akt - atypical protein kinase C isoforms λ and ζ are activated, leading in the case of skeletal muscle and WAT via Akt substrate of 160 kDa (AS160) and Rab guanosine triphosphatase (Rab-GTPase) to GLUT4 translocation and glucose uptake (e.g. 208).

Failure of insulin to promote its proper function is considered as insulin resistance of the affected tissue/cell. Proper insulin signalling may be impaired at any step, via phosphorylation of inhibitory sites, de-phosphorylation of activating sites, post-translational modification of enzymes, transcriptional repression of genes, or other events. FA, lipid intermediates like DAG or ceramides, inflammatory cytokines, adipokines and even high glucose levels may interfere with insulin signalling. Thereby, several pathways converge at different points. For example, TNFα, via its receptor, activates JNK1 which in turn is able to phosphorylate serine sites at IRS. DAG, either via lipolytic pathway or TAG formation is able to activate PKC θ, which acts on IRS as well. Some fatty acids have been shown to bind to toll-like receptors (TLRs) of which TLR4 activates IKK which impacts ceramide synthesis which subsequently may act on Akt phosphorylation by protein phosphatase 2A or PKC ζ. In this sense it seems that not TG per se, but rather lipid intermediates are in part responsible for or contributing to IR (207; 208).

Hence a complex interplay between signals derived from WAT in the form of cytokines and adipokines activating or reinforcing immune response as well as diverse lipid intermediates derived from altered lipolytic or lipogenetic function of adipocytes and hepatocytes play a role in developing, manifesting and aggravating the state of tissue IR. However, the exact time-dependent signalling events and the contribution of both inflammation and lipids solely or in concert remain incompletely understood.

4.12. Hypotheses and aims

Given the information above, the purpose of this thesis was to shed further light on the function of WAT in two different research settings in rodent models: First, to better understand its role and inter-organ crosstalk in a short-term overfeeding setting and the subsequent development of insulin resistance. And second, to use a WAT transplant-model (75) to understand the possible role of endogenous graft properties and site-specific effects in auto-regulating WAT mass.

For the first study, 12 week old male C57BL/6J mice were overfed for four days with a caloric dense HFD or a control chow diet. Additionally, animals with adipocyte-specific deletion of Fas/CD95, a known factor to influence inflammation, were used in this setting to further specify the role of WAT inflammation in the early development of IR. We hypothesized that even at such early stage, inflammation occurring in WAT plays a role in the development of hepatic insulin resistance and that adipocyte-specific Fas knock-out (AFasKO) animals would be protected from that development which was shown in a long-term setting (209). Further details and results are summarised under “project 1”.

For the second study, preliminary unpublished data suggested differential regulation of fat mass. Therefore, we used our previously implemented transplantation model (75) to search for factors released by WAT or liver or via inter-organ crosstalk which may regulate endogenous WAT mass after site-specific transplantation of exogenous donor WAT. For that purpose, eWAT from littermate donors was transplanted to the intestine (portal-transplanted animals, pTx) of C57BL/6J mice and, subsequently, the impact on body and WAT mass as well as on metabolic markers was studied. We hypothesized that WAT transplantation site would increase WAT total mass. This may be due to (a) factor(s) released by the transplant and/or neural innervation which either directly provide feedback to central regions or via portal drainage feed back from the liver to the brain and hence would influence food intake, energy expenditure, or main regulators of WAT expansion. Further details and preliminary results are summarised under “project 2”.

5. Results

Project 1:

5.1 Adipose tissue inflammation contributes to short-term high-fat diet-induced hepatic insulin resistance.
Michael Wiedemann, Stephan Wueest, Flurin Item, Eugen J. Schoenle, Daniel Konrad
Am J Physiol Endocrinol Metab 305: E388–E395, 2013.

5.2 Short-term HFD does not alter lipolytic function of adipocytes.
Michael Wiedemann, Stephan Wueest, Alexandra Grob, Flurin Item, Eugen J. Schoenle, Daniel Konrad
Adipocyte 3:2, 1–6; April/May/June 2014

Project 2:

5.3 Portal vein-drained adipose tissue transplants increase endogenous adipose tissue mass
Michael Wiedemann, Stephan Wueest, Eugen J. Schoenle, Daniel Konrad
Unpublished

5.1. Adipose tissue inflammation contributes to short-term high-fat diet-induced hepatic insulin resistance

Authors: Michael Wiedemann, Stephan Wueest, Flurin Item, Eugen J. Schoenle, Daniel Konrad

Journal: *Am J Physiol Endocrinol Metab* 305: E388–E395, 2013.

DOI: 10.1152/ajpendo.00179.2013.

PMID: 23736545

Contribution: Design, performance, analysis and interpretation of experiments Fig. 1A, E, Fig. 2B, C, Fig. 3A, B, Fig. 4A-C, Fig. 5A, E, Fig. 6B, C; editing and revising the manuscript.

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Adipose tissue inflammation contributes to short-term high-fat diet-induced hepatic insulin resistance

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Wiedemann MS, Wueest S, Item F, Schoenle EJ, Konrad D. Adipose tissue inflammation contributes to short-term high-fat diet-induced hepatic insulin resistance. *Am J Physiol Endocrinol Metab* 305: E388–E395, 2013. First published June 4, 2013; doi:10.1152/ajpendo.00179.2013.—High-fat feeding for 3–4 days impairs glucose tolerance and hepatic insulin sensitivity. However, it remains unclear whether the evolving hepatic insulin resistance is due to acute lipid overload or the result of induced adipose tissue inflammation and consequent dysfunctional adipose tissue-liver cross-talk. In the present study, feeding C57Bl6/J mice a fat-enriched diet [high-fat diet (HFD)] for 4 days induced glucose intolerance, hepatic insulin resistance (as assessed by hyperinsulinemic euglycemic clamp studies), and hepatic steatosis as well as adipose tissue inflammation (i.e., TNFα expression) compared with standard chow-fed mice. Adipocyte-specific depletion of the antiapoptotic/anti-inflammatory factor Fas (CD95) attenuated adipose tissue inflammation and improved glucose tolerance as well as hepatic insulin sensitivity without altering the level of hepatic steatosis induced by HFD. In summary, our results identify adipose tissue inflammation and resulting dysfunctional adipose tissue-liver cross-talk as an early event in the development of HFD-induced hepatic insulin resistance.

adipose tissue-liver cross-talk; lipotoxicity; diabetes mellitus

IN OBESITY, ADIPOSE TISSUE EXPANSION is accompanied by local infiltration of different types of inflammatory cells (3). The emerging cross-talk between infiltrating inflammatory cells and local cells, such as adipocytes, results in altered adipokine as well as increased proinflammatory cytokine production and secretion. Consequently, insulin resistance evolves both locally as well as systemically, e.g., in the liver, due to the evolving dysfunctional interorgan crosstalk (6, 8). Consistently impinging on adipose tissue inflammation improves obesity-associated insulin resistance (19, 20, 22, 25). Thus, adipose tissue inflammation may trigger hepatic insulin resistance potentially via the release of cytokines and/or lipids into the circulation.

In susceptible mouse strains, obesity and associated insulin resistance may be induced by a fat-enriched diet. It was demonstrated recently that a short period of high-fat diet (HFD), i.e., for 3–4 days, is sufficient to induce hepatic steatosis, hepatic insulin resistance, and adipose tissue inflammation (7, 13). However, it remains unclear whether hepatic insulin resistance (and hepatic steatosis) induced by a short bout of HFD is the result of acute lipid overload or whether it is at least partly mediated by adipose tissue inflammation, as it was demonstrated for long-term HFD feeding.

To assess a potential contribution of adipose tissue inflammation to short-term HFD-induced insulin resistance, we performed experiments in wild-type and adipocyte-specific Fas-knockout mice. We provide evidence that a short challenge of HFD triggers adipose tissue inflammation and hepatic insulin resistance in wild-type mice. Moreover, interfering with adipose tissue inflammation via adipocyte-specific Fas depletion preserved hepatic insulin sensitivity, suggesting that adipose tissue inflammation contributes to HFD-induced hepatic insulin resistance as early as 4 days after initiation of HFD.

MATERIALS AND METHODS

Animals. C57BL/6J mice were obtained from The Jackson Laboratory. Adipocyte-specific Fas-knockout mice ($Fas^{\Delta adipo}$) were generated as follows; mice with exon IX of Fas flanked with LoxP sites [a gift of Dr. A. Chervonsky, University of Chicago, and produced as described elsewhere (23)] were crossed with mice expressing Cre recombinase controlled by the Fabp4 promoter [B6.Cg-Tg(Fabp4-cre)1Rev/J; purchased from The Jackson Laboratory]. All mice were genotyped as described (25). Experiments presented in Figs. 1–3 were performed in $Fas^{f/f}$ mice. In all experiments, including $Fas^{\Delta adipo}$, the latter were compared with $Fas^{f/f}$ control littermates. In total, 94 mice were used for this study.

Twelve-week-old male mice were fed ad libitum with standard rodent diet (chow) or HFD (D12331; Research Diets, New Brunswick, NJ) for 4 days. HFD consisted of 58% of calories derived from fat, 25.5% from carbohydrate, and 16.5% from protein. Mice were fasted for 5 h prior to euthanization. All protocols conformed to the Swiss animal protection laws and were approved by the Cantonal Veterinary Office in Zurich, Switzerland.

Intraperitoneal glucose tolerance test. Mice were injected intraperitoneally with 2 g/kg body wt glucose after overnight fasting, as described previously (11). Blood glucose concentration was measured with a Glucometer (Accu-Check Aviva; Roche Diagnostics, Rotkreuz, Switzerland) with blood from tail-tip bleedings.

Hyperinsulinemic euglycemic clamp studies. Hyperinsulinemic euglycemic clamp studies with an insulin infusion rate of 18 $mU \cdot kg^{-1} \cdot min^{-1}$ were performed as described (17). Clamps were performed in freely moving mice. Glucose infusion rate was calculated once glucose infusion reached a more or less constant rate with blood glucose levels at 5 mmol/l (80–90 min after the start of insulin infusion). Thereafter, blood glucose was kept constant at 5 mmol/l for 15–20 min, and glucose infusion rate was calculated. The glucose disposal rate was calculated by dividing the rate of [3-^{3}H]glucose infusion by the plasma [3-^{3}H]glucose-specific activity (4, 9). Endogenous glucose production during the clamp was calculated by subtracting the glucose infusion rate from the glucose disposal rate (4, 9). Insulin-stimulated glucose disposal rate was calculated by subtracting basal endogenous

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glucose production (equal to basal glucose disposal rate) from glucose disposal rate during the clamp (18).

Determination of plasma insulin, free fatty acid, and triglyceride levels. Blood was sampled in mice fasted for 5 h. Plasma free fatty acid and triglyceride levels were determined as described elsewhere (25). Plasma insulin levels were measured using an ELISA kit as described previously (11).

Western blotting. For assessing insulin-stimulated Akt phosphorylation in liver, insulin (1 U/kg) was injected intraperitoneally in mice fasted for 5 h. Liver samples were harvested 15 min after insulin injection, snap-frozen in liquid nitrogen and stored at −80°C until homogenization. Liver samples or isolated adipocytes for Fas determination were homogenized as described previously (24). Protein concentration was determined using BCA assay (Pierce, Rockford, IL), and equivalent amounts of protein (50–75 μg) were resolved by LDS-PAGE (4–12% gel, NuPAGE; Invitrogen). Proteins were electrotransferred onto nitrocellulose membranes (0.2 μm; Bio-Rad, Reinach, Switzerland), and equal loading was confirmed by Ponceau S staining. The following primary antibodies were used: anti-Fas (Upstate, Lake Placid, NY), anti-actin (Millipore, Zug, Switzerland), and anti-phospho-Akt (Ser^{473}; Cell Signaling Technology, Danvers, MA). Membranes were exposed in an Image Reader and analyzed with Image Analyzer (FujiFilm, Dielsdorf, Switzerland). Arbitrary values obtained with Image Analyzer were normalized to an average of 1 in the control group.

RNA extraction and quantitative RT-PCR. Total RNA was extracted from epididymal or mesenteric adipose tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen, Basel, Switzerland), and concentration was determined spectrophotometrically (Nanodrop 1000; Nanodrop Technologies, Boston, MA). The integrity of each mesenteric fat RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (both Agilent Technologies, Basel, Switzerland). An RNA integrity number >8.0 was considered as acceptable for further processing; 0.75–1 μg of RNA was reverse transcribed with Superscript III Reverse Transcriptase (Invitrogen, Basel, Switzerland) using a random hexamer primer (Invitrogen). Taqman (Applied Biosystems, Rotkreuz, Switzerland) was used for real-time PCR amplification. The following PCR primers (Applied Biosystems) were used: TNFα Mm00443258_m1, IL-6 Mm00446190_m1, cd11b Mm00434455_m1, cd11c Mm00498698_m1, F4/80 Mm00802529_m1, MCP-1 Mm00441242_m1, IL-10 Mm00439614_m1, CD36 Mm00432403_m1, CD8 Mm00438116_m1, IFNγ Mm01168134_m1, MIP-1α Mm99999057_m1, and Ela3b Mm00840378_m1. Relative gene expression was obtained after normalization to 18sRNA (Applied Biosystems), using the formula $2^{-\Delta\Delta C_P}$ (16).

Total liver lipid determination. Liver tissue (10–30 mg) was homogenized in PBS, and lipids were extracted in a chloroform-methanol (2:1) mixture. Total liver lipids were determined by a sulfophosphovanillin reaction, as described previously (10).

Histology. Liver tissues were fixed in 4% buffered formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin.

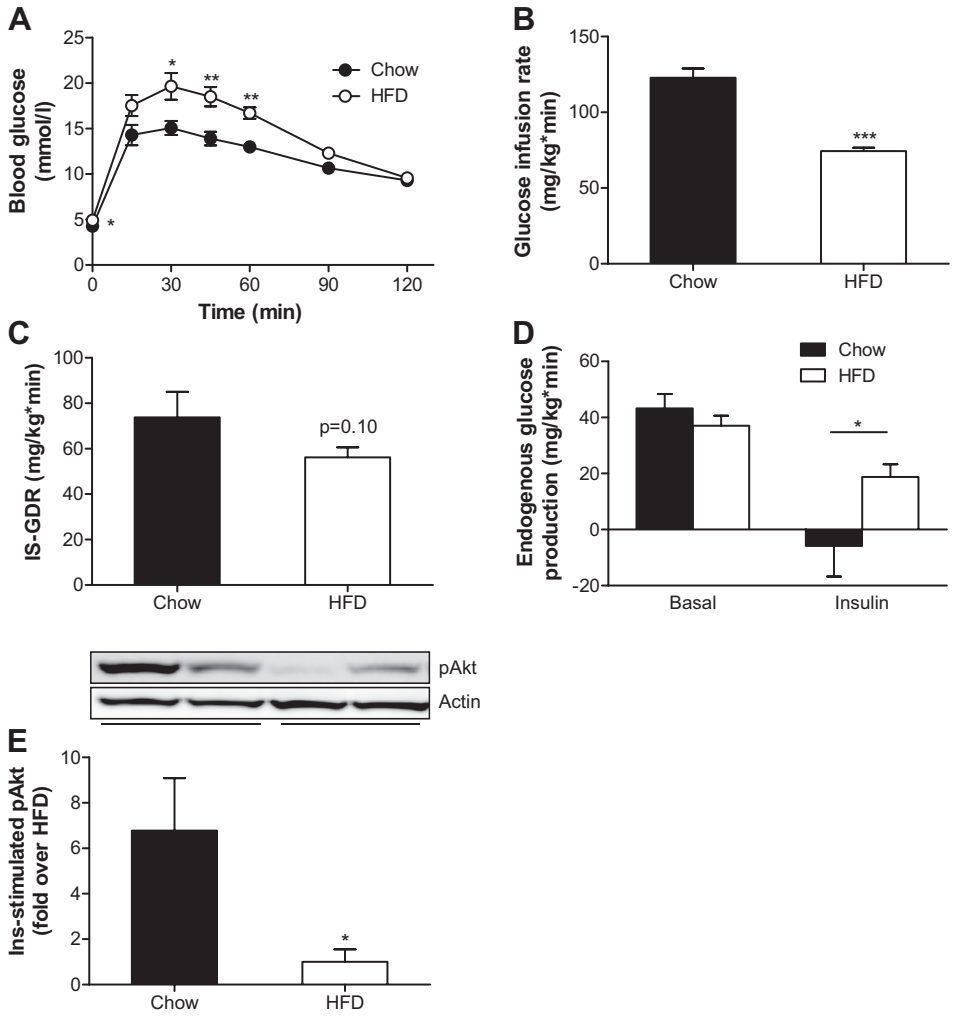

Fig. 1. Four days of high-fat diet (HFD) induces hepatic insulin resistance. *A*: intraperitoneal glucose tolerance test (2 g/kg body wt) was performed in chow-fed (●) and HFD-fed mice (○). Results are the means of 5 animals/group. *B–D*: hyperinsulinemic euglycemic clamp studies with glucose infusion rate, insulin-stimulated glucose disposal rate (IS-GDR), and basal as well as insulin-inhibited endogenous glucose production (EGP) were conducted in chow-fed (black bars) and HFD-fed mice (open bars). Results are means of 3 (chow-fed) or 9 (HFD-fed) animals. *E*: insulin (1 U/kg) was injected intraperitoneally (ip) in mice fasted for 5 h. Liver samples were harvested 15 min later, and phosphorylation of Akt at Ser^{473} was determined by Western blotting. A representative blot is shown at the *top*; $n = 3–4$. $^*P < 0.05$ (Student's *t*-test). All error bars represent SE. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (Student's *t*-test).

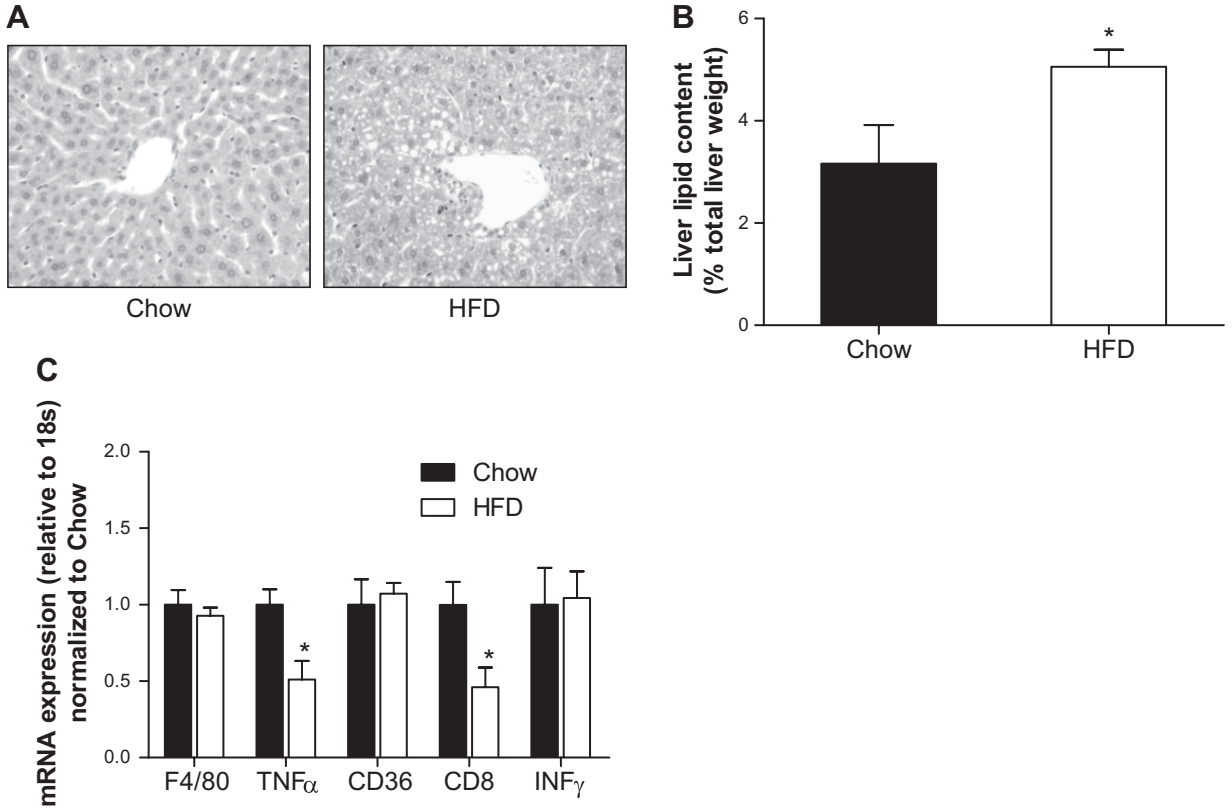

Fig. 2. Four days of HFD induces liver steatosis. *A*: representative hematoxylin and eosin-stained liver sections from chow-fed (*left*) and HFD-fed (*right*) mice (magnification ×20). *B*: total liver lipids were determined and expressed relative to liver weight. Results are the means of 4 (chow-fed) or 8 (HFD-fed) animals. *C*: mRNA expression of respective genes was analyzed in liver of chow-fed (black bars) and HFD-fed (open bars) mice; $n = 6–9$. All error bars represent SE. *$P <$ 0.05 (Student's *t*-test).

Data analysis. Statistical analyses were performed using Student's *t*-test. *P* values <0.05 were considered significant. All error bars represent SE.

RESULTS

Four days of HFD induces hepatic insulin resistance in C57BL6/J mice. To determine a potential impact of short-term HFD on glucose metabolism, intraperitoneal glucose tolerance test was performed in 12-wk-old mice receiving either a 58 cal% HFD for 4 days or a standard chow diet. As depicted in Fig. 1*A*, glucose tolerance was deteriorated significantly in HFD-fed littermates [area under the curve (AUC) in HFD-fed mice 1,761 ± 78 mmol·l^{-1}·min vs. AUC in chow-fed mice 1,433 ± 50 mmol·l^{-1}·min; $P < 0.01$]. Of note is that body weight was similar between both groups (26.4 ± 1.0 g in HFD-fed mice vs. 25.6 ± 1.2 g in chow-fed animals; *P* =

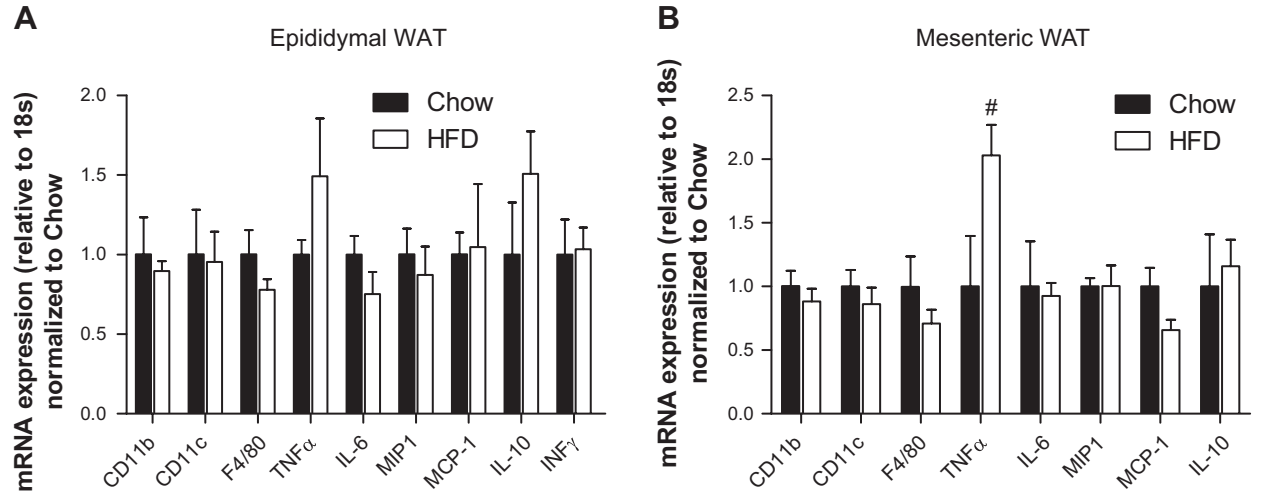

Fig. 3. Four days of HFD increases adipose tissue TNFα expression. mRNA expression of respective genes was analyzed in epididymal white adipose tissue (WAT; *A*) or mesenteric WAT (*B*) of chow-fed (black bars) and HFD-fed (open bars) mice; $n = 3–11$. All error bars represent SE. #*P* = 0.05 (Student's *t*-test).

0.55). To assess insulin sensitivity, hyperinsulinemic euglycemic clamp studies were performed. Glucose infusion rate was decreased significantly in HFD-fed mice, reflecting reduced total body insulin sensitivity (Fig. 1*B*). Whereas insulin-stimulated glucose disposal rate was not significantly diminished (Fig. 1*C*), insulin-mediated inhibition of endogenous (mainly reflecting hepatic) glucose production was reduced in HFD-fed mice (Fig. 1*D*), suggesting that a short bout of HFD impacts mainly on hepatic insulin sensitivity. Similarly, insulin-stimulated Akt phosphorylation was reduced significantly in HFD compared with chow-fed mice (Fig. 1*E*). Of note is that total Akt protein levels were not different between the groups (data not shown).

Four days of HFD induces liver steatosis. Hepatic steatosis is strongly associated with insulin resistance; however, it is presently unclear whether insulin resistance causes hepatic steatosis or whether the increase in triglycerides or lipid metabolites causes the development of hepatic insulin resistance. As depicted in Fig. 2, *A* and *B*, 4 days of HFD was sufficient to increase hepatic lipid accumulation, as assessed by histological examinations and biochemical determination of total liver lipid content. In contrast, short-term high-fat feeding had no major impact on liver inflammation, as determined by mRNA expression of inflammatory as well as macrophage markers (Fig. 2*C*). Of note is that liver TNFα expression was reduced upon HFD, which is in accordance with previously published data reporting a trend toward decreased TNFα liver transcription after short bouts of high-fat feeding (13). Reduced CD8 mRNA expression may suggest lower T cell infiltration in livers of HFD-fed mice.

Four days of HFD increases adipose tissue TNFα expression. Previously, 3–4 days of HFD was reported to induce adipose tissue inflammation (7, 12, 13). Therefore, we determined mRNA expression of proinflammatory cytokines and macrophage markers in epididymal as well as mesenteric adipose tissue. As shown in Fig. 3, *A* and *B*, TNFα expression was increased significantly in mesenteric fat pads and trendwise in epididymal fat pads of HFD-fed compared with chow-fed mice. Of note is that harvesting of mesenteric adipose tissue is not that simple and is often contaminated by pancreatic tissue (2). To make sure that a pure fraction of mesenteric adipose tissue was analyzed, expression of Ela3b was determined. All samples included expressed Ela3b at a very low level confirming pure fraction of mesenteric adipose tissue. Therefore, as reported previously (13), a short bout of HFD was sufficient to induce TNFα expression in adipose tissue.

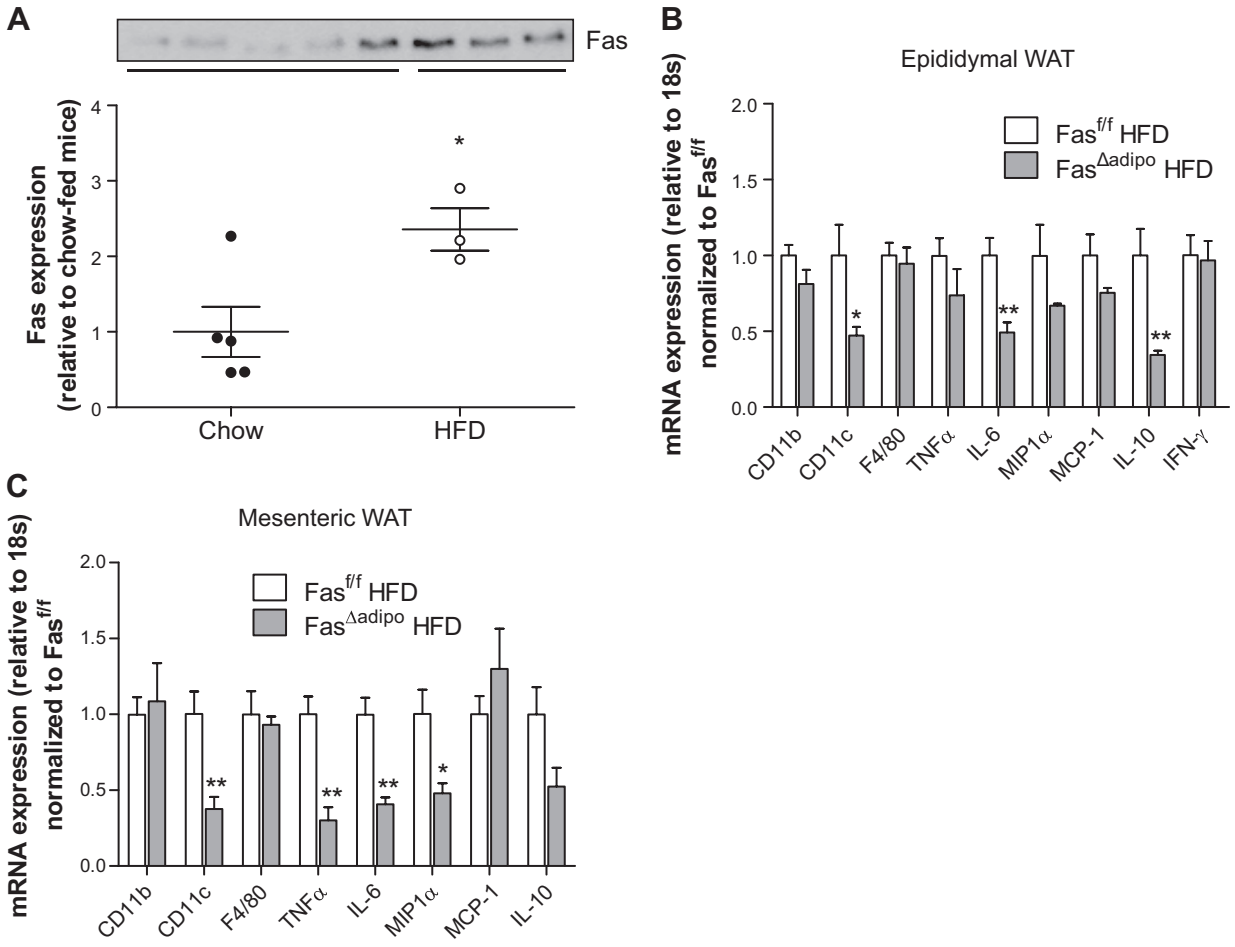

Fig. 4. Adipocyte-specific Fas-knockout mice are protected from HFD-induced adipose tissue inflammation. *A*: total cell lysates were prepared from isolated epididymal adipocytes harvested from wild-type C57Bl6/J mice fed either a chow diet or HFD for 4 days. Lysates were resolved by LDS-PAGE and immunoblotted with anti-Fas antibody; $n = 3–5$. *B* and *C*: mRNA expression of respective genes was determined in epididymal (*B*) or mesenteric WAT (*C*) of HFD-fed $Fas^{f/f}$ (open bars) and $Fas^{\Delta adipo}$ (gray bars) mice; $n = 5–6$. All error bars represent SE. $^*P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test).

Table 1. *Phenotypic characteristics of HFD-fed Fas$^{f/f}$ and Fas$^{\Delta adipo}$ mice*

	Fas$^{f/f}$	Fas$^{\Delta adipo}$
Body weight, g	27.6 ± 0.5	28.5 ± 0.4
Epididymal fat mass, mg	288.6 ± 33.6	304.4 ± 21.3
Mesenteric fat mass, mg	248.4 ± 21.3	269.4 ± 23.2
Blood glucose, mmol/l	8.8 ± 0.2	9.3 ± 0.3
Insulin, pmol/l	0.85 ± 0.30	1.06 ± 0.22
FFA, mmol/l	0.58 ± 0.10	0.65 ± 0.11
TG, mg/dl	78.1 ± 10.4	104.3 ± 18.5

Results are means ± SE of 6–25 mice fasted for 5 h. FFA, free fatty acids; TG, triglycerides. Differences between both groups were statistically not significant.

HFD-fed adipocyte-specific Fas-knockout mice express decreased levels of proinflammatory cytokines in white adipose tissue. In the present study, we aimed to determine whether adipose tissue inflammation contributes to HFD-induced hepatic insulin resistance as early as 4 days after initiation of high-fat feeding. Previously, we found that adipocyte-specific Fas-knockout mice (Fas$^{\Delta adipo}$) were protected from long-term HFD-induced insulin resistance (25). Importantly, adipose tissue inflammation was reduced in Fas$^{\Delta adipo}$ mice and was associated with improved hepatic insulin sensitivity. Therefore, if adipose tissue inflammation contributes to short-term HFD-induced hepatic insulin resistance, Fas$^{\Delta adipo}$ mice may be a good model to study the presence of such cross-talk. As depicted in Fig. 4*A*, 4 days of HFD increased Fas protein content significantly in isolated adipocytes of wild-type mice. Moreover, expression of proinflammatory cytokines and macrophage markers was reduced significantly in both epididymal as well as mesenteric pat pads of Fas$^{\Delta adipo}$ mice challenged by a short bout of HFD compared with control littermates (Fig. 4, *B* and *C*), suggesting that Fas depletion diminished adipose tissue inflammation. Further phenotypic characteristics of HFD-fed Fas$^{f/f}$ and Fas$^{\Delta adipo}$ mice are described in Table 1.

Adipocyte-specific Fas-knockout mice are protected from short-term HFD-induced hepatic insulin resistance. We next assessed glucose metabolism in HFD-fed adipocyte-specific Fas-knockout and control mice. As depicted (Fig. 5*A*), glucose tolerance was improved significantly in HFD-fed Fas$^{\Delta adipo}$ mice compared with control mice (AUC in Fas$^{\Delta adipo}$ mice 1,564 ± 66 mmol·l^{-1}·min vs. AUC in Fas$^{f/f}$ mice 1,717 ± 42 mmol·l^{-1}·min; $P < 0.05$). In hyperinsulinemic euglycemic clamp studies, glucose infusion rate was not different between Fas$^{f/f}$ and Fas$^{\Delta adipo}$ mice (Fig. 5*B*). In contrast to insulin-stimulated glucose disposal rate, which was not different between the two groups

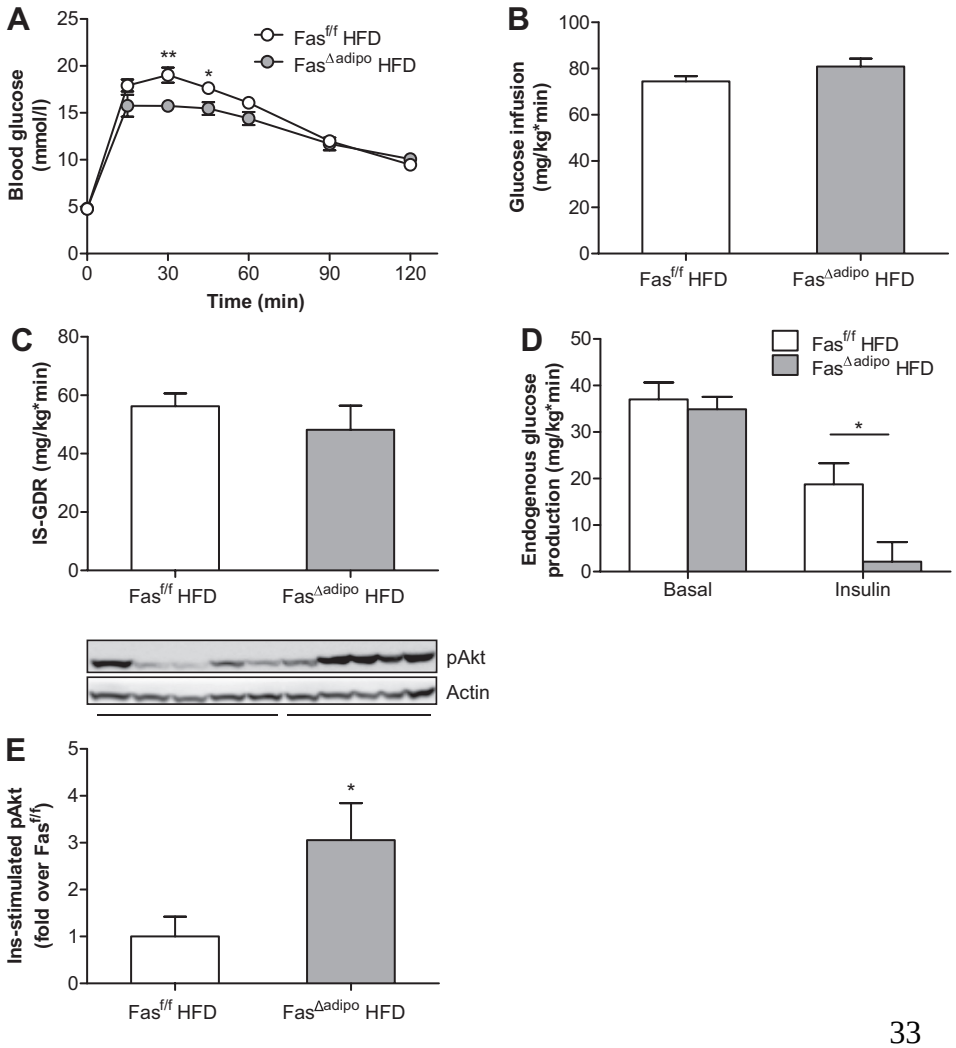

Fig. 5. Adipocyte-specific Fas-knockout mice are protected from HFD-induced hepatic insulin resistance. *A*: intraperitoneal glucose tolerance test (2 g/kg body wt) was performed in HFD-fed Fas$^{f/f}$ (○) and Fas$^{\Delta adipo}$ mice (gray circles). Results are the means of 11 (Fas$^{f/f}$) or 8 animals (Fas$^{\Delta adipo}$). *B–D*: hyperinsulinemic euglycemic clamp studies with glucose infusion rate, IS-GDR, and basal as well as insulin-inhibited EGP were conducted in HFD-fed Fas$^{f/f}$ (open bars) and Fas$^{\Delta adipo}$ mice (gray bars). Results are the means of 9 (Fas$^{f/f}$) or 7 animals (Fas$^{\Delta adipo}$). *E*: insulin (1 U/kg) was injected ip in mice fasted for 5 h. Liver samples were harvested 15 min later, and phosphorylation of Akt at Ser^{473} was determined by Western blotting. Western blot is shown at the *top*; $n = 5$/group. All error bars represent SE. *$P < 0.05$; **$P < 0.01$ (Student's *t*-test).

(Fig. 5*C*), insulin-mediated inhibition of hepatic glucose production was almost completely preserved in $Fas^{\Delta adipo}$ mice, whereas it was clearly blunted in Fas-expressing littermates (Fig. 5*D*). Similarly, insulin-stimulated Akt phosphorylation was sustained in livers of $Fas^{\Delta adipo}$ mice compared with control mice (Fig. 5*E*). Of note, total Akt protein levels were not different between the groups (data not shown). Thus, adipocyte-specific Fas-knockout mice are protected from HFD-induced hepatic insulin resistance.

Adipocyte-specific Fas depletion has no impact on liver lipid accumulation. In contrast to improved hepatic insulin sensitivity, total liver lipid content was not different between HFD-fed adipocyte-specific Fas-knockout and control mice (Fig. 6, *A* and *B*). Similarly, expression of several inflammation markers was similar between both groups (Fig. 6*C*). Thus, Fas depletion in adipocytes protects mice from developing short-term HFD-induced adipose tissue inflammation and hepatic insulin resistance but not hepatic steatosis.

DISCUSSION

The present study suggests that adipose tissue inflammation contributes to short-term HFD-induced hepatic insulin resistance. Such a notion is based on the following findings: *1*) 4 days of HFD induces adipose tissue TNFα expression; *2*) glucose intolerance as well as hepatic insulin resistance develops 4 days after initiation of HFD; and *3*) Fas depletion specifically in adipocytes reduces both HFD-induced adipose tissue inflammation as well as hepatic insulin resistance.

Several lines point toward a role of adipose tissue inflammation in the development of hepatic insulin resistance in obesity. It is thought that increased production and release of proinflammatory cytokines from visceral, i.e., omental and mesenteric adipose tissue into the portal vein, may contribute to the development of hepatic insulin resistance (6). A role for such dysfunctional fat-liver cross-talk in the pathogenesis of obesity-associated hepatic insulin resistance is supported by the fact that impinging on adipose tissue inflammation protects against HFD-induced insulin resistance and steatosis (15, 20, 25). Our findings presented here suggest that adipose tissue inflammation occurs early on in the course of HFD-induced metabolic alterations and contributes to hepatic insulin resistance. Such results seem to be in contrast to previous findings claiming that short-term HFD-induced insulin resistance is independent of inflammation (13). Although this study demonstrates induction of adipose tissue inflammation as manifested by an increase in macrophage infiltration of adipose tissue as well as by increased expression of proinflammatory cytokines (mainly TNFα), it fails to show a beneficial effect of macrophage and lymphocyte depletion as well as of hematopoietic cell-specific Jun NH_2-terminal kinase (JNK)-deficiency on short-term HFD-induced insulin resistance in mice (13). In contrast, we find herein a favorable effect of adipocyte-specific Fas deficiency on both adipose tissue inflammation and hepatic insulin sensitivity. Of note, differences in TNFα expression were stronger in mesenteric compared with epididymal adipose tissue, supporting the "portal theory." The latter proposes that the liver is exposed directly to increas-

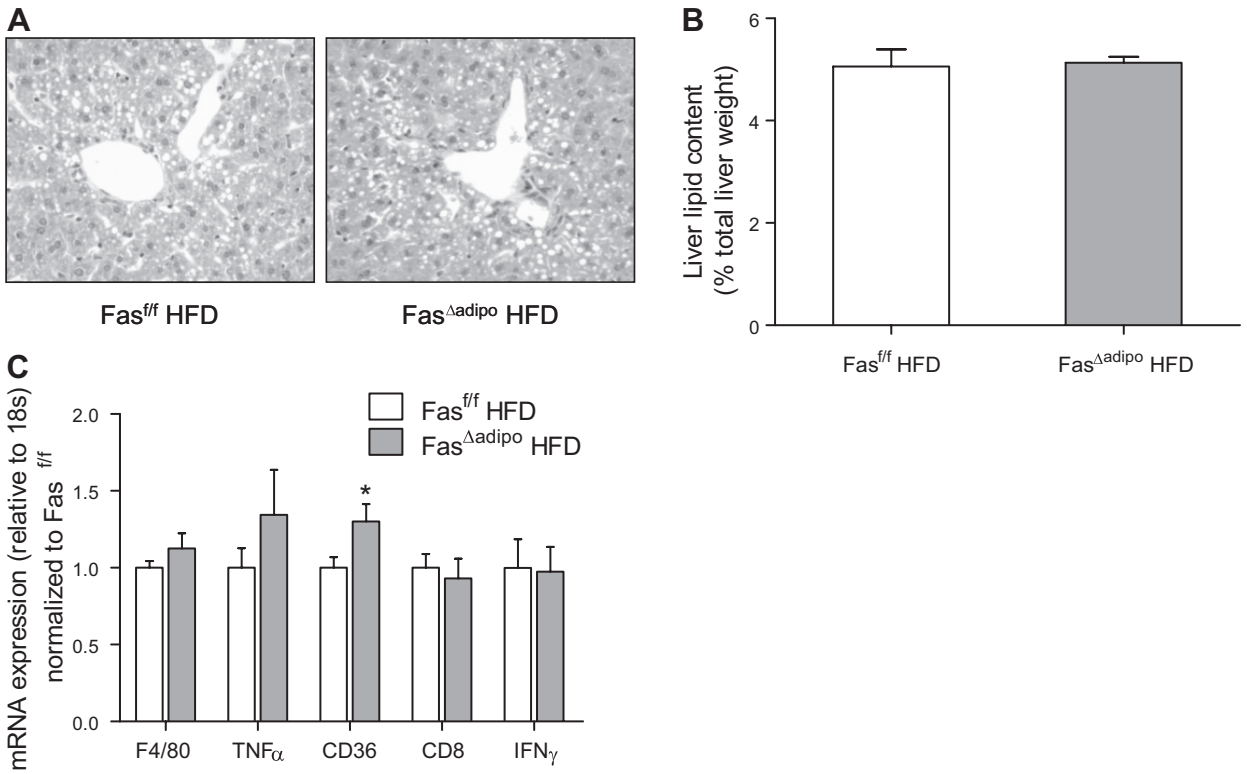

Fig. 6. Adipocyte-specific Fas depletion has no impact on liver lipid accumulation. *A*: representative hematoxylin and eosin-stained liver sections from HFD-fed $Fas^{f/f}$ (*left*) and $Fas^{\Delta adipo}$ (*right*) mice (magnification ×20). *B*: total liver lipids were determined and expressed relative to liver weight. Results are the means of 8 ($Fas^{f/f}$) or 5 HFD-fed animals ($Fas^{\Delta adipo}$). *C*: mRNA expression of respective genes was determined in liver of HFD-fed $Fas^{f/f}$ (open bars) and $Fas^{\Delta adipo}$ mice (gray bars); $n = 9$. All error bars represent SE. $^*P < 0.05$ (Student's *t*-test).

ing amounts of free fatty acids and/or proinflammatory factors released from visceral fat into the portal vein, promoting the development of hepatic insulin resistance (6). Our findings presented here may suggest that short-term HFD-increased TNFα expression in adipose tissue induces hepatic insulin resistance. In support of such a notion, TNFα was shown recently to induce insulin resistance in hepatocytes in culture (5). Moreover, it was reported that inhibition of TNFα by the monoclonal antibody infliximab improves HFD-induced hepatic insulin resistance in rats (1).

There is a strong association between hepatic steatosis and insulin resistance, although it is still debated whether insulin resistance is the cause of hepatic steatosis or whether the increase in triglycerides (or of lipid metabolites such as ceramides, diacylglycerol, and acyl-CoAs) causes the development of hepatic and/or systemic insulin resistance (14). In this regard, our observation that $Fas^{\Delta adipo}$ mice were protected from hepatic insulin resistance but not hepatic steatosis is intriguing and indicates that the latter may be the result of an acute lipid overload rather than a dysfunctional adipose tissue-liver cross-talk at an early stage of HFD. Moreover, it may suggest that short-term HFD-induced hepatic steatosis develops independently of concomitant hepatic insulin resistance. Supporting this notion, it was reported previously that 3 days of HFD induced hepatic insulin resistance and steatosis as well as hepatic inflammation and Kupffer cell activation. Interestingly, depletion of the latter improved hepatic insulin sensitivity, whereas it did not affect hepatic steatosis (12). Conversely, Samuel et al. (21) reported in rats that treatment with 2,4-dinitrophenol normalized short-term HFD-induced hepatic steatosis, whereas it improved only partly the ability of insulin to suppress endogenous glucose production. Of note is that $Fas^{\Delta adipo}$ mice depicted both improved hepatic insulin sensitivity as well as reduced total liver lipid content after 6 wk of HFD (25), suggesting that, later in the course of HFD-induced obesity, adipose tissue inflammation and its resulting dysfunctional adipose tissue-liver cross-talk may impact on both hepatic insulin sensitivity as well as hepatic steatosis.

In conclusion, our results identify adipose tissue inflammation and consecutive dysfunctional adipose tissue-liver cross-talk as an early event in the development of HFD-induced deterioration of hepatic insulin sensitivity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, exist for any of the authors.

AUTHOR CONTRIBUTIONS

M.S.W., S.W., and F.I. performed the experiments; M.S.W., S.W., F.I., and D.K. analyzed the data; M.S.W., S.W., F.I., and D.K. interpreted the results of the experiments; M.S.W., S.W., F.I., and D.K. prepared the figures; M.S.W., S.W., E.J.S., and D.K. edited and revised the manuscript; M.S.W., S.W., F.I., E.J.S., and D.K. approved the final version of the manuscript; S.W. and D.K. contributed to the conception and design of the research; D.K. drafted the manuscript.

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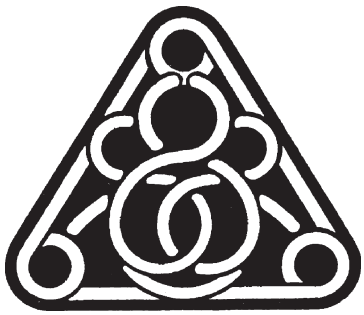

5.2. Short-term HFD does not alter lipolytic function of adipocytes

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Short-term HFD does not alter lipolytic function of adipocytes

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Keywords: adipose tissue–liver crosstalk, lipotoxicity, diabetes mellitus, fat depot, lipolysis

A short bout of high fat diet (HFD) impairs glucose tolerance and hepatic insulin sensitivity. We recently identified adipose tissue inflammation and resulting dysfunctional adipose tissue–liver cross-talk as an early event in the development of HFD-induced hepatic insulin resistance. In particular, reducing white adipose tissue (WAT) inflammation by adipocyte-specific depletion of Fas/CD95 protected mice from developing hepatic insulin resistance but not hepatic steatosis. Herein, we expanded our previous work and determined the impact of four days of HFD on lipolytic activity of isolated adipocytes. Compared with chow-fed mice, the degree of basal and isoproterenol-stimulated free fatty acid (FFA) and glycerol release was similar in HFD-fed animals. Moreover, insulin's ability to suppress lipolysis remained intact suggesting retained insulin sensitivity. Despite unaltered lipolysis, circulating FFA concentrations were greatly increased in non-fasted HFD-fed mice. In conclusion, a short-term HFD challenge does not affect lipolytic function of adipocytes. The observed increase of circulating FFA levels in randomly fed animals may rather be the result of increased dietary fat supply.

Three to four days of HFD suffice to induce hepatic insulin resistance, hepatic steatosis, and adipose tissue inflammation in susceptible mouse strains.[1-4] However it remains debatable whether the acute dietary lipid overload, increased FFAs mobilized from endogenous fat depots, or inflammatory cytokines—all known to have causal roles in above mentioned processes per se—are responsible for the observed metabolic changes. We recently provided evidence that adipose tissue inflammation and an early dysfunctional crosstalk between adipose tissue and liver participates in the development of hepatic insulin resistance.[5] To this end, adipocyte-specific Fas/CD95-deficient ($Fas^{\Delta adipo}$) or backcrossed control ($Fas^{f/f}$) mice on a C57BL/6J background were fed either standard chow or HFD (12% or 58% of calories derived from fat) for 4 d. Besides its well-known role in the regulation of apoptosis, Fas/CD95 can also induce non-apoptotic pathways.[6] In particular, Fas activation mediates the secretion of pro-inflammatory cytokines[7,8] and its adipocyte-specific expression may contribute to obesity-induced adipose tissue inflammation, hepatic insulin resistance as well as hepatic steatosis.[9] Feeding mice a HFD for four days induced Fas protein level in isolated adipocytes, mesenteric adipose tissue inflammation (i.e., TNFα expression), hepatic steatosis, and hepatic insulin resistance as assessed by hyperinsulinemic–euglycemic clamp.[5] Of note, adipocyte-specific Fas depletion relieved adipose tissue inflammation and hepatic insulin resistance without altering the degree of hepatic steatosis. Accordingly, it was recently demonstrated that TNFα release from WAT explants was increased after three days of HFD feeding and that TNFα neutralization prevented the development of hepatic insulin resistance.[1] These data suggest that adipose tissue inflammation and resulting dysfunctional adipose tissue-liver crosstalk, at least partly mediated via TNFα, is an early event in the development of HFD-induced insulin resistance. Moreover, the observation that improved hepatic insulin sensitivity in adipocyte-specific Fas knockout mice was not associated with decreased hepatic steatosis[5] would suggest that hepatic fat accumulation after a short bout of high fat feeding plays no major role in the development of hepatic insulin resistance and that adipose tissue inflammation and hepatic steatosis are two distinct processes in the development of hepatic insulin resistance.

Previously, we observed that eight weeks of HFD increased basal lipolysis and blunted insulin's ability to inhibit FFA release from isolated epididymal adipocytes.[10,11] Herein, we aimed to investigate whether four days of HFD were sufficient to affect lipolysis. We first determined body weight and adipose tissue mass of mice fed either a chow or a HFD for 4 d. Contrary to other reports investigating the effect of short-term HFD,[1,2,4] we did not observe any impact of 4 d of HFD on body weight gain (chow-fed mice 0.2 ± 0.2 g, n = 22; HFD-fed mice 0.6 ± 0.2 g, n = 12; P = 0.16) or adipose tissue mass (**Fig. 1A**). Similarly, fasting glucose (chow-fed mice 8.1 ± 0.4 mmol/l, n = 14; HFD-fed mice 8.3 ± 0.3 mmol/l, n = 11; P = 0.70) and fasting insulin levels (chow-fed mice 0.67 ± 0.20 ng/ml, n = 6; HFD-fed mice 0.85 ± 0.27 ng/ml, n = 6; P = 0.61) were not different between the

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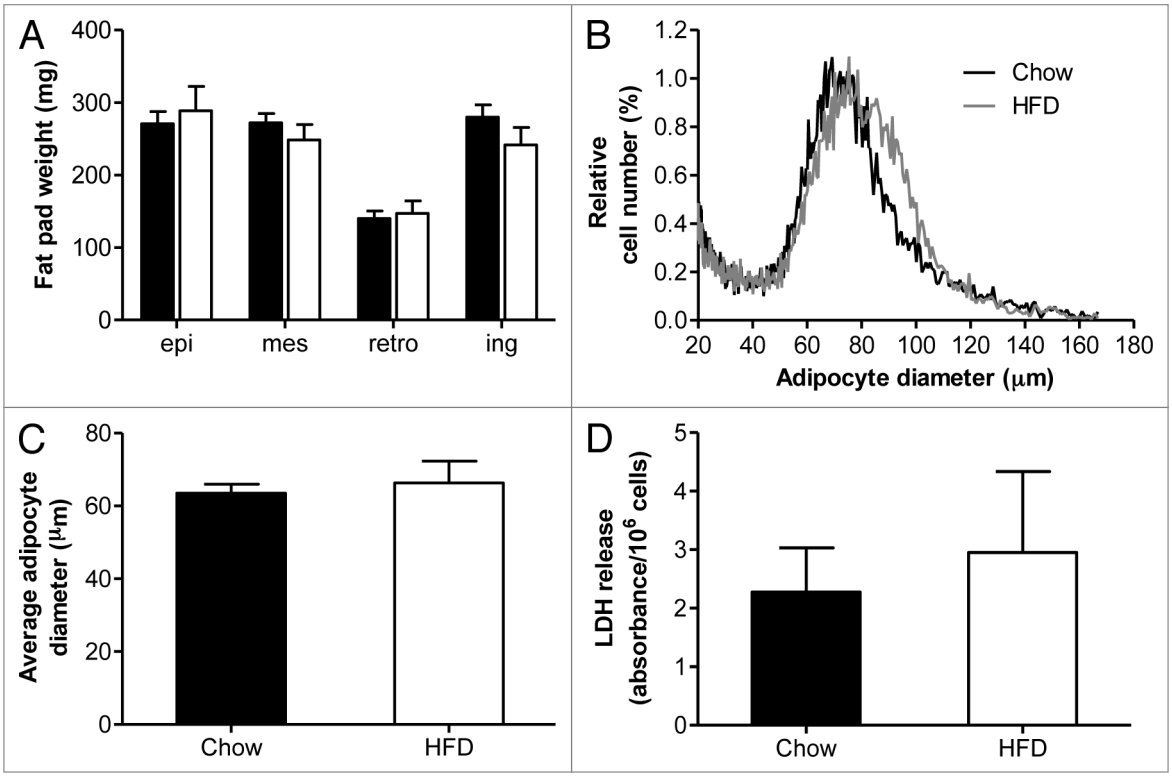

Figure 1. Similar fat pad weights and adipocyte size in chow- and short-term HFD-fed mice. (**A**) Fat pad weight of different adipose depots was determined in chow- (black bars) and short-term HFD-fed (open bars) mice. Results are the means ± SEM of 11–14 mice. Epi, epididymal; mes, mesenteric; retro, retroperitoneal; ing, inguinal. Adipocyte cell size distribution of isolated epididymal adipocytes (**B**), mean adipocyte diameter (**C**), and viability of isolated adipocytes determined via a lactate-dehydrogenase assay (**D**) are depicted for chow-fed (black bars) and HFD-fed (open bars) non-fasted mice. Results are the means ± SEM of 4 independent experiments.

groups. Moreover, neither distribution of adipocyte according to their cell size (**Fig. 1B**) nor average adipocyte diameter (**Fig. 1C**) was different between chow- and HFD-fed mice. Importantly, release of lactate dehydrogenase (LDH) was similar suggesting comparable viability of isolated adipocytes from chow-fed and HFD-fed animals (**Fig. 1D**).

Lipolytic activity of epididymal adipocytes isolated from fed mice was assessed next. As depicted in **Figure 2A and B**, neither basal nor isoproterenol-stimulated lipolysis did differ between chow-fed and HFD-fed animals. In addition, the fold increase in isoproterenol-induced FFA release (chow 6.9 ± 1.8-fold vs. HFD 8.0 ± 2.9-fold; $P = 0.75$) and glycerol release (chow 4.9 ± 0.8-fold vs. HFD 4.2 ± 0.5-fold; $P = 0.43$) was similar, suggesting alike sensitivity to isoproterenol. Comparable basal and isoproterenol stimulated FFA and glycerol release suggested a similar degree of re-esterification. Indeed, calculations according to Rosenstock et al.[12] revealed similar degrees of re-esterification for both basal (chow 33.7 ± 12.5% vs. HFD 51.5 ± 9.8%; $P = 0.30$) and isoproterenol-stimulated (chow 17.3 ± 4.9% vs. HFD 24.3 ± 6.2%; $P = 0.41$) lipolysis in both groups. Moreover, the ability of insulin to inhibit lipolysis (**Fig. 2C**) and to stimulate Akt phosphorylation (**Fig. 2D**) remained intact in adipocytes of HFD-fed mice suggesting no deterioration of insulin sensitivity in the latter. Even though lipolytic activity was similar between chow- and HFD-fed animals, circulating FFA levels were significantly increased in the latter in the fed state (**Fig. 2E**) whereas glycerol and triglyceride levels were not different (**Fig. 2F and G**). Similarly, increased FFA levels were previously reported for short-term HFD-fed non-fasted rats.[13,14] However, after five hours of fasting FFA concentrations were similar between chow- and HFD-fed mice (**Fig. 2H**). Likewise, FFA levels were found to be no longer different between chow- and short-term HFD-fed animals after various periods of fasting,[13,15-17] i.e., circulating FFA levels did increase in chow-fed animals during fasting to match FFA concentrations observed in HFD-fed animals. Of note, similar levels of phospho-HSL and phospho-perilipin were found in epididymal WAT after 5 h of fasting (**Fig. 2I**) suggesting similar lipolytic activity of WAT in both groups.

The fact that lipolysis was not affected by a short bout of HFD would suggest that increased circulating FFA levels in the fed state of short-term HFD-fed mice are rather the result of increased dietary fat supply. Interestingly, a short period of HFD sufficed to induce hepatic steatosis[3,5,13] whereas it did not result in any significant increase in skeletal muscle and adipose fat accumulation as reported herein for adipose tissue and described previously for both skeletal muscle and/or adipose tissue.[13,18] It is conceivable that the development of hepatic fat accumulation results from increased FFA flux into hepatocytes due to a dietary-induced rise in circulating FFA levels in the postprandial state. In this regard, a similar fat composition between the liver and

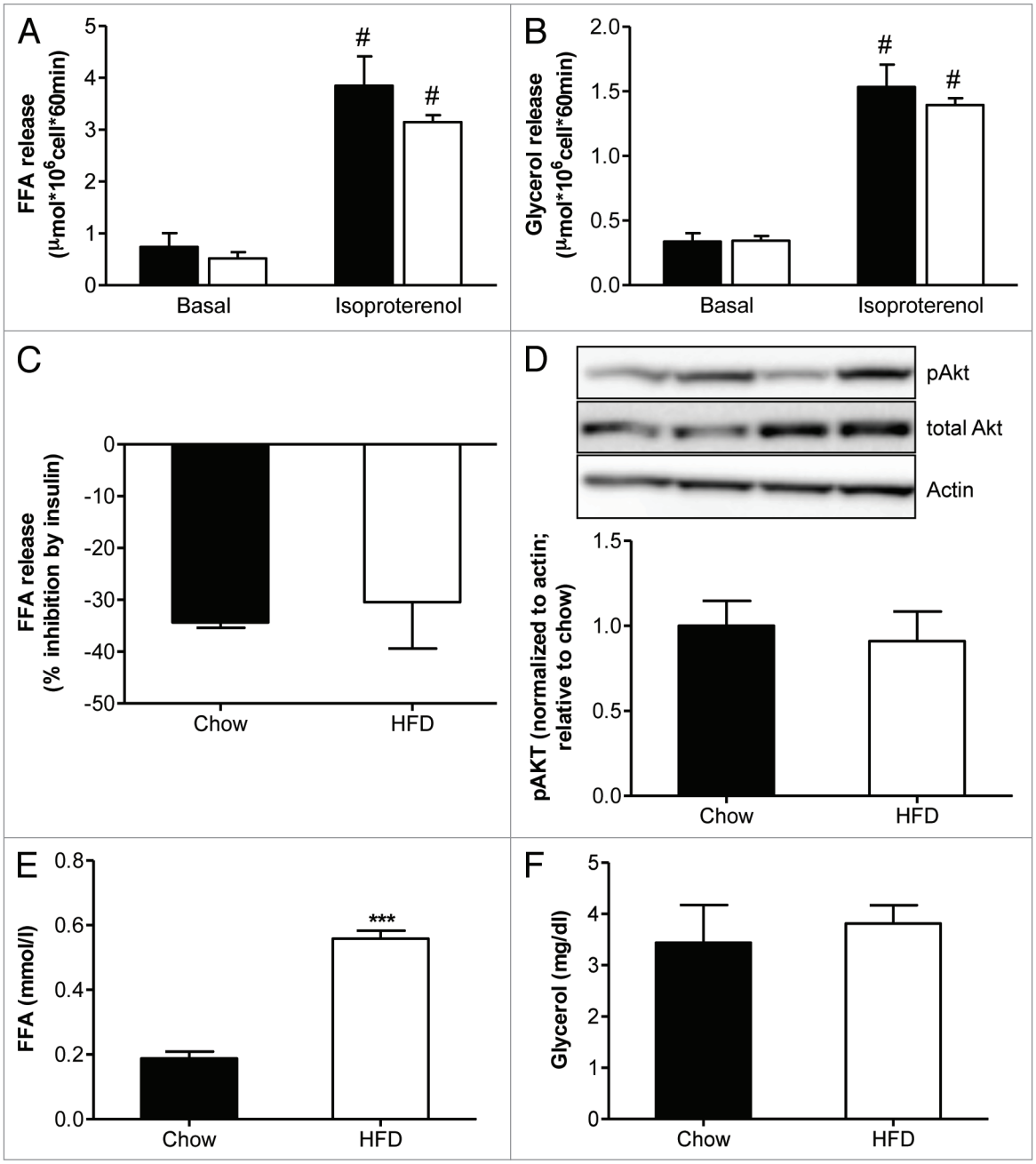

Figure 2A–G (For H–K, see page 4). Similar lipolytic activity but increased non-fasted circulating FFA levels in short-term HFD-fed mice. Basal, isoproterenol-stimulated (**A and B**), and insulin-inhibited (**C**) FFA or glycerol release from adipocytes isolated from epididymal WAT of chow-fed (black bars) and HFD-fed (open bars) mice are depicted. Results are the means ± SEM of 4 independent experiments in non-fasted mice. (**D**) Insulin (1 U/kg) was injected i.p. in randomly fed mice. Epididymal WAT was harvested 15 min later and phosphorylation of Akt at Ser473 was determined and normalized to actin ($n = 4$). Representative blots are shown. FFA (**E**), glycerol (**F**), as well as triglycerides (**G**) levels were determined in systemic blood plasma of non-fasted mice, $n = 4$. (**H**) FFA levels were determined in systemic blood plasma in mice fasted for 5 h, $n = 6$. (**I**) Phospho-HSL (Ser^{660}) and phospho-Perilipin (Ser^{522}) protein levels were determined in epididymal WAT harvested from chow-fed (black bars) and HFD-fed (open bars) mice fasted for 5 h ($n = 6$) and normalized to actin. Representative blots are shown. FATP1 (**J**) and GLUT4 (**K**) content was analyzed in epididymal WAT harvested from randomly fed mice ($n = 4$). Representative blots are shown. All error bars represent SEM $^{*}P < 0.05$ and $^{***}P < 0.001$ (Student t test), $^{\#}P < 0.05$ (isoproterenol vs. basal; Mann–Whitney U test).

diet, both having an abundance of 18:2 fatty acid, was previously demonstrated.[13] The evolving hepatic insulin resistance may then serve as early adaptation mechanism to protect the liver from further fat accumulation. As previously demonstrated we did not observe increased mRNA expression of macrophage or inflammatory markers in livers of mice fed a fat-enriched diet for four days. Moreover, as mentioned above, a reduction of adipose tissue inflammation had no impact on short-term HFD-induced

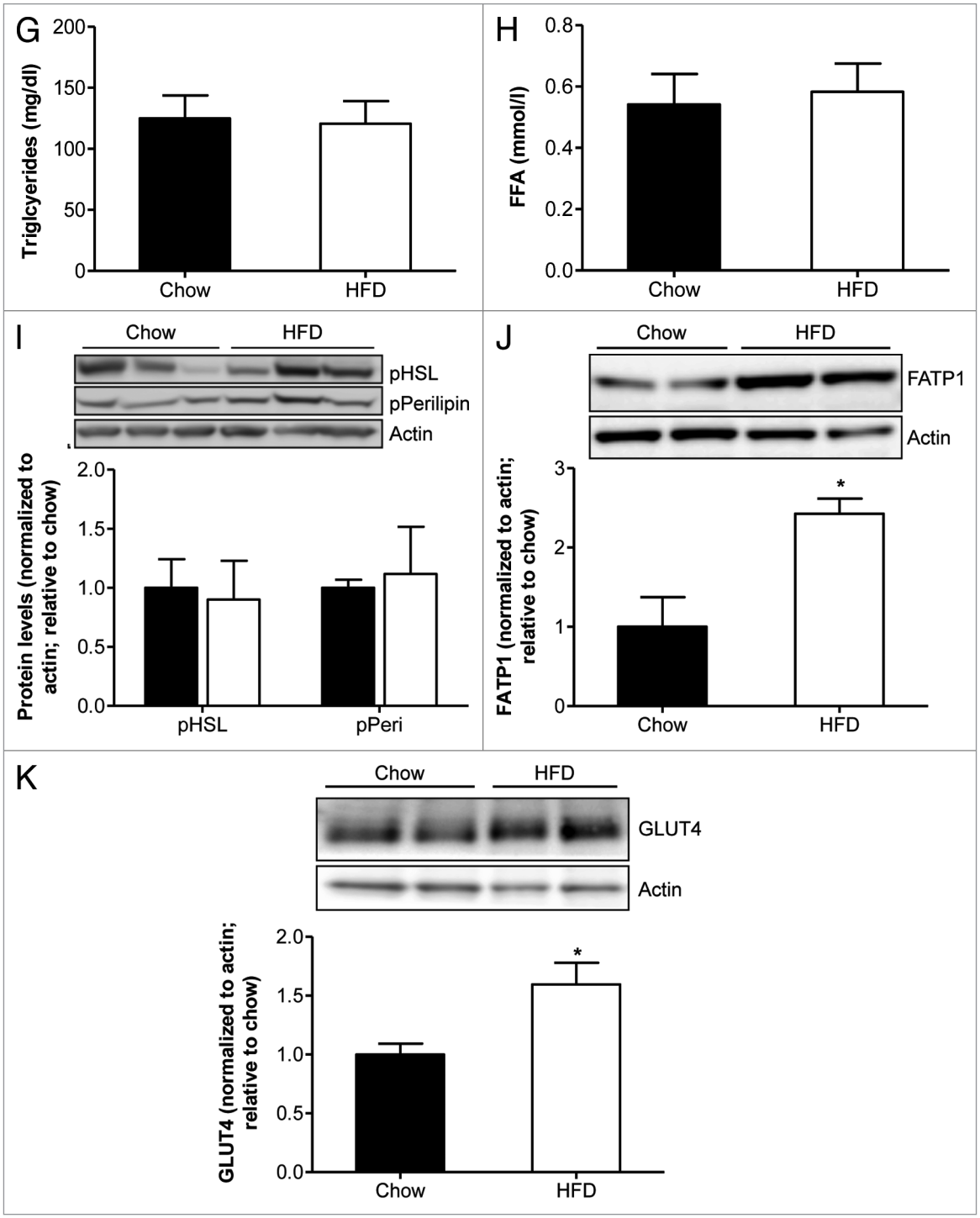

Figure 2G–K. For figure legend, see page 3.

hepatic steatosis but improved hepatic insulin sensitivity. Thus, such result would suggest that short-term HFD-induced hepatic insulin resistance is not the result of hepatic inflammation or hepatic fat accumulation. Of note, insulin sensitivity of skeletal muscle and adipose tissue was retained after four days of HFD as shown herein and as was previously reported.[1,3,5,13] The anticipated increase in circulating insulin levels due to hepatic insulin resistance[19] may enhance insulin-stimulated glucose uptake into adipose tissue and, thus, evolving fat accumulation.

Potentially, reduced fatty acid uptake by adipocytes may lead to increased circulating FFA levels (due to reduced FFA flux into adipocytes) and hence, to reduced re-esterification. However, as depicted in **Figure 2A**, FFA levels in the incubation media of adipocytes of chow- and HFD-fed mice were similar, as was the

degree of re-esterification. In addition, protein abundance of the long-chain fatty acid transporters FATP1 (ACSVL5) was even increased in adipose tissue of HFD-fed mice (**Fig. 2J**). Hence, such data would suggest that there is no impaired capacity of FFA uptake into adipocytes of HFD-fed mice. Likewise, basal and insulin-stimulated glucose uptake does not appear to be affected by short-term HFD as indicated by similar GLUT1 (not shown) and increased GLUT4 (**Fig. 2K**) content as well as preserved insulin-stimulated Akt phosphorylation (**Fig. 2D**).

In conclusion, short-term HFD does not affect lipolytic activity of isolated adipocytes. The observed increase of circulating FFA levels in randomly fed animals after short-term high fat feeding may rather be the result of increased dietary fat supply.

Materials and Methods

Animals

C57BL/6J mice were originally obtained from The Jackson Laboratory and then bred in our own facility. At the age of 12 wk animals were fed ad libitum with standard rodent diet (chow) or HFD (D12331, Research Diets) for 4 d. HFD consisted of 58% of calories derived from fat, 25.5% from carbohydrates, and 16.5% from protein. For additional experiments, mice were not fasted prior to sacrifice to be able to analyze adipocytes in a fed (baseline) state as there seems to be a rapid change e.g., in circulating FFAs with prolonged periods of fasting.[13] All protocols conformed to the Swiss animal protection laws and were approved by the Cantonal Veterinary Office in Zurich, Switzerland.

Determination of plasma glucose and insulin levels

Blood was sampled in mice fasted for 5 h. Blood glucose concentration was measured with a Glucometer (Accu-Check Aviva, Roche Diagnostics) with blood from tail-tip bleedings. Plasma insulin levels were measured using an ELISA kit as described previously.[20]

Viability assessment and cell size determination

Adipocytes were isolated and viability was determined with an LDH assay as described previously.[21] Aliquots of isolated adipocytes were used to determine mean cell diameters. Cell size was analyzed by a Multisizer™ 3 Coulter Counter® as follows. Two hundred microliters of isolated adipocytes were incubated in 2 ml of 2% osmium tetroxide in a collidine solution at 37 °C for 48 h. Cells were strained with a 250 μm nylon mesh and second with a 40 μm mesh and in-between fraction was counted.

Lipolysis assays

Lipolysis was assessed in isolated adipocytes as indicated. Isolated adipocytes were incubated in the absence or presence of 100 nM insulin or 1 μM isoproterenol (Sigma) for one hour.[21] FFA levels were measured using the ACS-ACOD-MEHA method from Wako Chemicals GmbH. Triglyceride and glycerol levels were determined using a colorimetric assay, as described.[9]

Western blotting

Samples were homogenized as described previously.[21] Protein concentration was determined using BCA assay (Pierce), and equivalent amounts of protein (30–40 μg) were resolved by LDS-PAGE (4–12% gel, NuPAGE; Invitrogen). Proteins were electrotransferred onto nitrocellulose membranes (0.2 μm; Bio-Rad) or PVDF membranes (0.45 μm; Roche Diagnostics GmbH), and equal loading was confirmed by Ponceau S staining. The following primary antibodies were used: anti-actin was purchased from Millipore, anti-phospho-HSL (Ser660) as well as anti-phospho-Akt (Ser473) and total Akt from Cell Signaling Technology, anti-FATP1 from Santa Cruz Biotechnology, and anti-phospho-Perilipin (Ser522) from Vala Sciences. Primary antibodies against GLUT1 and GLUT4 were a kind gift from Dr Amira Klip, The Hospital for Sick Children, Toronto, Canada. Membranes were exposed in an Image Reader and analyzed with Image Analyzer (FujiFilm). Arbitrary values obtained with Image Analyzer were normalized to an average of 1 in the control chow-fed group.

Data analysis

Statistical analyses were performed using the Student *t* test or Mann–Whitney U test where appropriate. P values < 0.05 were considered significant. All error bars represent SEM.

Disclosure of Potential Conflicts of Interest

No conflict of interest existed for any of the authors.

Acknowledgments

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5.3. Portal vein-drained adipose tissue transplants increase endogenous adipose tissue mass

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Contribution: Design, performance, analysis and interpretation of all experiments; writing, editing, and revising the manuscript.

Portal vein-drained adipose tissue transplants increase endogenous adipose tissue mass

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Abstract

White adipose tissue (WAT) is capable of auto-regulating its own mass as was demonstrated by its removal, its addition through transplantation or its removal and autologous replacement. Herein, we tested the hypothesis that increasing portal-drained WAT mass in normal chow-fed C57BL/6 mice leads to dys-regulation of total body fat mass. Four weeks after transplantation, endogenous fat pad mass was increased by approximately 20% in mice receiving a portal-drained fat transplant. Importantly, the impact on endogenous fat mass seemed to be dependent on the amount of fat transplanted since small transplants had no effect on endogenous fat pad regulation. Food intake, energy expenditure or activity parameters were not different between sham-operated and transplanted mice. The observed increase in endogenous fat mass can currently not be clearly attributed to either a hypertrophic or hyperplastic response, or both, and requests further mechanistic evaluation. Indirect evidence from former experiments, however, suggests rather a hyperplastic than a hypertrophic response of endogenous WAT depots in transplanted mice. Collectively, our findings may suggest a disturbed regulation of endogenous fat mass in mice receiving a portal vein-drained fat transplant.

Key words: adipose tissue, WAT, transplantation, tissue cross-talk

Introduction

White adipose tissue (WAT) was initially regarded solely as storage organ accumulating excess energy in form of triglycerides and releasing mainly free fatty acids when fuel is needed. During the past 20 years, however, it became evident that adipose tissue has endocrine functions mediated by the secretion of different adipokines, cytokines and fat-derived metabolites, which may act locally, i.e. in an autocrine/paracrine manner, or affect more distant organs such as the liver (1-3), and actively contribute to the regulation of energy balance (4).

Food intake, energy expenditure and fat mass are tightly regulated, reflecting powerful neuro-endocrine feedback circuits that mediate the crosstalk between the brain (where feeding and energy expenditure are controlled) and adipose tissue (where excess energy in stored). The first hormone to exemplify this system was the adipokine leptin, which is released in response to an increase in fat mass to inhibit food intake and to augment energy expenditure (5). Interestingly, obese patients develop (central) leptin resistance, potentially maintaining obesity by insufficient leptin-mediated inhibition of food intake and/or insufficient stimulation of energy expenditure (6). In addition to leptin, several other hormones and cytokines released from fat tissue, pancreas and the gut (incretins) transmit peripheral signals to the central nervous system in order to regulate food intake and energy expenditure, a system that when properly regulated acts to maintain body weight. Conversely, alterations in this tight regulation may result in obesity. Empirically, it is well known that some individuals are more prone to become obese whereas others are protected. It is conceivable that such susceptibility/resistance to obesity is linked to differences in these neuro-endocrine feedback loops.

There is increasing evidence that adipose tissue may not only be the main effector of such feedback mechanisms (by storing excessive energy), but may actively contribute to the control of food intake and energy expenditure. For example, removal of fat tissue on one site leads to compensatory augmentation of fat mass in other depots resulting in maintained overall fat mass (7). Moreover, Rooks et al. (8) demonstrated that subcutaneous transplantation of donor eWAT would lead to a reduction in endogenous WAT mass of the transplanted animals without major alterations in other parameters. In addition, we previously found that fat tissue transplantation in mice was accompanied by a slight decrease in endogenous

epididymal WAT mass (9) suggesting that the artificial increase in fat mass may induce compensatory mechanism in order to restore normal total fat mass and body weight. However, little is known about potential mechanism involved in fat mass auto-regulation. There is evidence that adipose tissue mass may be regulated either via humoral or neuronal signals originating from the hypothalamus (10; 11).

We previously developed a fat transplantation model in mice demonstrating a role for the transplantation site of WAT on glucose homeostasis (9; 12). Transplantation of epididymal WAT (eWAT) to a systemically drained acceptor site improved glucose tolerance in mice (9). In contrast, eWAT transplanted to a portal vein-drained acceptor site (pTx) deteriorated glucose tolerance and induced hepatic insulin resistance (IR) (12) providing direct evidence in support of the portal hypothesis. The latter proposes that the development of hepatic IR and liver steatosis results from direct exposure of the liver to increasing amounts of free fatty acids and pro-inflammatory factors released from visceral fat into the portal vein of obese patients (13). Preliminary observations from our studies suggested that not only insulin sensitivity but also other metabolic parameters are influenced by the site of transplantation. For example, endogenous epididymal WAT mass was slightly decreased in chow-fed mice receiving a systemically drained WAT transplant (9). In addition, we have evidence that this effect is dependent on the mass transplanted WAT. Herein, we explored the hypothesis that portal transplantation of eWAT alters endogenous WAT mass compared to sham operated animals and propose a potential role of adipose tissue-derived factors in the regulation of total body fat mass.

Research Design and Methods

Animals. Male C57BL/6JOlaHsd mice were purchased from Harlan Netherlands (Harlan, AD Horst, The Netherlands). All mice were housed in a pathogen-free environment on a 12-h light-dark cycle (lights on at 6am) and an ambient temperature of 22°C, with free access to standard rodent diet (diet 3430, Provimi Kliba, Kaiseraugst, Switzerland). Prior to sacrifice and tissue harvest, animals were fasted for 3 hours. Adipose tissue mass was evaluated from pooled samples of both epididymal, inguinal (iWAT), and retroperitoneal (rWAT) depots as well as total mesenteric depot per mouse. Transplanted fat pad weight was not included in

calculation. All protocols conformed to the Swiss animal protection laws and were approved by the Cantonal Veterinary Office in Zurich, Switzerland.

Surgical procedure. Transplantation procedure was performed at 8 weeks of age as described (12). Briefly, mice were anaesthetized with isoflurane (Abbott, Baar, Switzerland). Both epididymal fat pads (total transplanted fat: ~280 mg [range 160-400 mg]) were removed from the donor mouse, rinsed with 0.9% saline, and thereafter stitched to the mesenterium of the recipient mouse (portal transplantation, pTx) using Monocryl 6.0 (Johnson-Johnson, Spreitenbach, Switzerland). Sham-operated control mice underwent the same surgical procedure without receiving a fat transplant. Therefore, tissues were briefly exposed and gently moved with forceps and afterwards repositioned. Subcutaneous (nuchal fold) injection of buprenorphine every 6-8 h for at least two days or until necessary was used for analgesia. To prevent transplant rejection, donor and recipient mice were littermates. In general, all animals tolerated surgery well. Subsequent analyses were done 4-5 weeks post transplantation. In total, 30 different litters in 7 cohorts were analysed. As proper re-vascularisation and drainage site can only be evaluated post-mortem, several animals had to be excluded due to double-drainage, i.e. at least one of the grafts was clearly re-vascularised and attached to any site not draining to the portal vein (e.g. inner side of peritoneum or endogenous epididymal fat pad). These animals and their corresponding litter sham control animals were taken out completely from all analyses and evaluated separately (data not shown). Sham and pTx numbers are not equal as sometimes two pTx animals were operated from a litter of 5 animals.

Indirect calorimetry. For metabolic phenotyping, mice were individually housed in plexiglass air-tight cages designed for open circuit calorimetry (PhenomasterTM, TSE Systems GmbH, Bad Homburg, Germany). Prior to starting measurement, animals were separated to accustom to single-caging for 48 hours and body weight was monitored twice daily. The system was calibrated prior to each run according to manufacturer's instruction with known weights for food and drinking water as well as known gas concentrations for oxygen uptake and carbon dioxide production measurements. Sampling interval was 2 min per cage every 18 min measuring 8 cages in a row and one reference cage and automatically recorded using the integrated software package. For time-course analysis, data were binned in 24 one

hour values. Locomotor activity data was recorded with a 2-dimensional infrared beam grid. Food and water intake were continuously monitored to the nearest 0.01 gram or millilitre. Any visible residual spilled food pellets found in the cage after measurement period were weighed and deducted. Animals were fed standard chow with a metabolizeable energetic density of 3.14 kcal/g (3430, Provimi Kliba AG, Kaiseraugst, Switzerland). Data were collected for four 24-hour days. The first day was analysed separately, however, included in time course and cumulative analysis. In general, most values obtained were stable from day 2 to day 4 suggesting a steady-state in measured parameters. Values are expressed per mouse per day unless otherwise stated (14; 15). The first two lots of animals were only tested for a single day of metabolic cages, the 3rd and 4th lot for 2 days and the 5th and 6th lot for 4 consecutive days. Hence number of animals per day reduces and is indicated in the corresponding figures.

Western blotting. Liver, eWAT and mWAT tissue were harvested and snap-frozen in liquid nitrogen and stored at -80°C until homogenization. Tissue samples were pulverized in liquid nitrogen and homogenized in a buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1% NP-40, 0.25% sodium deoxycholate, 1mM sodium vanadate, 1mM NaF, 10mM sodium β-glycerophosphate, 1 mM sodium pyrophosphate, 0.2 mM PMSF and a 1:1000 dilution protease inhibitor cocktail (P8340, Sigma Aldrich). Protein concentration was determined using BCA assay (Pierce, Rockford, IL), and equivalent amounts of protein (20–40 µg) were resolved by LDS-PAGE (4–12% gel, NuPAGE; Invitrogen) or self-casted 6-10% SDS gels. Proteins were electrotransferred onto nitrocellulose membranes (0.2 µm; Bio-Rad, Reinach, Switzerland) or PVDF membranes (0.45 µm; Roche Diagnostics GmbH, Mannheim, Germany), and equal loading was confirmed by Ponceau S staining. Membranes were blocked for one to two hours in 5% non fat dry milk (BioRad) resolved in Tris buffered saline, containing 0.1% Tween 20. Membranes were incubated over night at 4°C on a rocking platform with respective primary antibodies. The following primary antibodies were used: anti-pACCSer79, anti-ACC, anti-pHSLSer660, anti-ATGL, anti-PPARγ (all from Cell Signaling Technology, Danvers, MA), anti-SREBP-1c, anti-CEBPβ, anti-CEBPα (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-pPerilipinSer522 (Vala Sciences, San Diego, CA), and anti-actin (Millipore, Zug, Switzerland). Subsequently, membranes were incubated with

appropriate secondary antibody (horseradish peroxidase-conjugated; Santa Cruz Biotechnology) for 45 min at 37°C and 45 min at room temperature. Bands were detected after 5-10 minute incubation with Lumi-Light substrate (Roche) and subsequently exposed in an Image Reader and analyzed with Image Analyzer (FujiFilm, Dielsdorf, Switzerland). Protein expression was normalized to loading control actin.

RNA extraction and quantitative RT-PCR. Total RNA was extracted from eWAT, iWAT, and mWAT as well as liver using the RNeasy Lipid Tissue Mini Kit (Qiagen, Basel, Switzerland), and concentration was determined spectrophotometrically (Nanodrop 1000; Nanodrop Technologies, Boston, MA). The integrity of each RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000NanoLabChip kit and a bioanalyzer 2100 (both Agilent Technologies, Basel, Switzerland). An RNA integrity number >8.0 was considered as acceptable for further processing; 0.75–1 µg of RNA was reverse transcribed with Superscript III Reverse Transcriptase (Invitrogen, Basel, Switzerland) using a random hexamer primer (Invitrogen). Taqman (Applied Biosystems, Rotkreuz, Switzerland) was used for real-time PCR amplification. The following PCR primers (Applied Biosystems) were used: Srebf1_Mm00550338_m1, Insig1_Mm00463389_m1, Fgf21_Mm00840165_g1, Ucp1_Mm01244861_m1, Cidea_Mm00432554_m1. Gene expression was calculated with standard curves included on each plate and is expressed relative to loading control 18sRNA (Applied Biosystems) and normalized to sham-operated animals.

Microarray Experiment Description

cDNA preparation. The quality of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDropTechnologies, Delaware, USA) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). Only those samples with a 260 nm/280 nm ratio between 1.8–2.1 and a 28S/18S ratio within 1.5–2 were further processed.

cDNA preparation was performed using the Ambion WT Expression Kit (P/N 4411973) in a following manner: RNA samples (100 ng) were reverse-transcribed into double-stranded cDNA. Double-stranded cDNA was in vitro transcribed to cRNA. cRNA was purified using Nucleic Acid Binding Beads in a manner described in a protocol. cRNA yield and size distribution were determined using NanoDrop ND 1000 and Bioanalyzer 2100. Sense-strand cDNA was synthesized by reverse transcription

of 10 µg of cRNA using random primers. RNase H was used to degrade cRNA template leaving single-stranded sense-strand cDNA. The sense-strand cDNA was purified using Nucleic Acid Binding Beads. The yield of cDNA was determined using NanoDrop ND 1000.
Fragmentation and labeling was performed using AffymetrixGeneChip WT Terminal Labling Kit (P/N 901525) as described here. Fragmentation reaction with 5.5 µg of cDNA was followed by the labeling of the fragmented single-stranded cDNA in the presence of biotinylated nucleotides and terminal deoxynucleotidyltransferase (TdT) that catalyzes nontemplate-directed nucleotide incorporation onto the 3'-OH end of a single-stranded DNA.

Array hybridization. Biotin-labeled cDNA samples were mixed in 150 µl of WT Hybridization Cocktail (Affymetrix, P/N 901667) containing a Hybridization Controls and Control Oligonucleotide B2 (Affymetrix, P/N 900454). Samples were hybridized to GeneChip® Mouse Gene 2.1ST Array Strip (Affymetrix, P/N 901628) in the GeneAtlas Hybridization Station for 20 h at 48°C. Arrays were then washed using AffymetrixGeneAtlas Fluidics Station. An AffymetrixGeneAtlas Imaging Station was used to measure the fluorescent intensity emitted by the labeled target.

Analsis of microarray data. The resulting CEL files have been analyzed using Bioconductor/R. In particular the steps included RMA summarization (16), quality control in order to check the similarity of samples within the groups and the distinction between the groups and then pairwise comparisons between groups. In the case of t-test in pairwise comparisons, the false discovery rate was controlled using Benjamini-Hochberg correction (17). A p-value of 0.1 for trendwise and 0.05 for significant changes together with a fold-change of 1.4 was taken as cut-off value between both groups for gene expression analysis.

Data analysis. Data are presented as mean ± standard deviation. *General analysis*: Statistical analyses were performed using Student's t-test or Mann-Whitney U test where appropriate. Log transformation was used if data were not normalized. *Metabolic cage analysis*: As energy expenditure is dependent on body mass, an ANCOVA was used with body mass (BM) as co-variable (14; 18; 19). Additionally, as weight stability is assumed during evaluation of parameters in metabolic cages (e.g.

20), delta BM (∆BM) was used as a second co-variable for statistical evaluation. Animals BM during metabolic cage evaluation remained, however, quite stable (sham = -0.38 ± 0.54 g vs. pTx = -0.3 ± 0.76 g) and hence ∆BM co-variable analysis revealed no further major adjustments and is therefore omitted from results. For clarity, relevant data are presented with and without correction. P values <0.05 were considered significant. The software in use for graphical analysis was Prism Version 5.0 (GraphPad Software Inc.,San Diego, CA, USA). Statistical evaluation was done with SPSS 21 (IBM).

Results

Endogenous WAT mass is increased in pTx transplanted mice

At the time of surgery, body mass did not differ between pTx and sham-operated mice. Additionally, body mass gain from time of surgical intervention until sacrifice was similar between both groups (Table 1). Of note, body mass in these chow-fed animals was not related to total fat pad mass, neither in total nor in sub-group analyses (Fig.1 A-C). In contrast, endogenous adipose tissue mass defined by the four indicated depots differed between the groups. There was a significant increase in total endogenous WAT mass in pTx vs. sham-operated animals (Table 1). Subgroup analysis of the four depots showed significant differences for mWAT and a strong trend for eWAT (Fig. 1 D). Of note, mass of transplanted fat pads decreased by about 30% from 277±66 mg to 188±51 mg. Therefore, portal transplantation of WAT increases endogenous fat mass in acceptor mice.

No differences in food intake and energy expenditure between pTx and sham-operated mice

To assess potential differences in food intake and/or energy expenditure, mice were housed in metabolic cage units for up to 96 hours. As depicted in Table 1, neither food intake (FI), nor energy expenditure (EE), nor activity differed significantly between both groups on any day of evaluation. Additionally, day-night patterns did not show any alterations between the two groups (data not shown). Time course analysis over sampling period is demonstrated in Figures 2 A to C. There seems to be a diverging trend between the groups when evaluated over longer periods. Unfortunately, we could not prolong housing time in metabolic cages to study

whether the curves of cumulative FI would continue spreading as time of single cage housing was limited to a total of 6 days including adaptation for ethical reasons. Additionally, number of animals per day decreased (see methods section) and therefore may have biased our result due to the small cohort sampled.

Microarray data

Since mWAT and eWAT mass differed between both groups (Fig. 1), these two tissues were screened with microarray analysis for potential gene candidates, which could explain observed differences in phenotype. Genes that were altered between pTx and sham-operated mice are depicted in Table 2 and Table 3 for eWAT and mWAT, respectively. We did not observe major differences for any known pathways involved in regulating WAT mass between both groups. Manual inspection of raw data did not identify any further genes mainly due to high intra-group variation for some potential targets. A fold change of 1.4 (2^0.5) was considered as relevant between the two groups. However, Benjamini-Hochberg corrected FDR setting of 5% revealed no single gene to be altered in either tissue. Metacore® pathway analysis revealed no consecutive pathways to be altered.

Similar expression of Ucp1, Cidea, Fgf21 and Insig1 in eWAT of pTx and sham-operated mice

Due to unproductive microarray analysis, we focused on some targets previously reported to contribute to browning (appearance of brown adipocytes in WAT leading to increased energy expenditure) of iWAT or to WAT mass regulation. We did not observe any differences in Ucp-1 or Cidea expression in iWAT between both groups excluding differences in its browning capacity and matching results obtained for energy expenditure (Table 1). In addition, Fgf21, which was shown to be increased in obese humans and patients with T2DM (21) and found to control energy expenditure and insulin action in mice (22; 23), was expressed at very low levels in liver and did not differ between both groups. However, its role may be more complex with different functions in human and mouse (24). Moreover, the Insig1-SREBP1-SCD1 pathway was recently reported to regulate WAT mass (25). As depicted in Fig. 3 A-C, Insig1 and Srebf1 expression did not differ between pTx and sham-operated mice.

No alterations of protein level in eWAT of pTx mice

Phosphorylation state or total protein levels of major regulators of fatty acid (FA) and/or triglyceride (TG) metabolism was assessed next. Neither phosphorylation state of hormone-sensitive lipase (HSL) and perilipin, nor total protein concentration of adipose triglyceride lipase (ATGL) was altered (Fig. 4). Similarly, phosphorylation of Acetyl-CoA carboxylase (ACC), which is the main regulator for Malonyl-CoA production, or its total protein level did not differ between both groups (Fig 4). Next master regulators of WAT development were assessed. Unexpectedly, neither peroxisome proliferator-activated receptor γ (PPARγ) nor any of the other main up- or downstream regulators like CCAAT/enhancer-binding protein alpha (CEBPα) or beta (CEBPβ), or sterol regulatory element-binding protein 1c (SREBP-1c) showed any difference at protein level.

Discussion

The present study sought to explore the effect of portal vein-drained white adipose tissue transplantation on whole body energy metabolism and its regulatory effect on endogenous white adipose tissue mass. It demonstrates that exogenously transplanted portal drained white adipose tissue is increasing endogenous WAT mass in chow-fed mice by yet unknown mechanisms. No differences in food intake, energy expenditure, or activity between sham-operated and pTx mice were observed. Similarly, no gross changes in endogenous WAT's gene expression profile explaining the observed phenotype could be identified. Neither could alterations in protein levels or phosphorylation states thereof be identified to explain the increased endogenous eWAT mass.

The increase in endogenous WAT mass could have been the result of increased food intake or decreased energy expenditure; however, none of these parameters varied significantly between pTx and sham-operated mice during the four-day observation period. Such finding does not rule out the possibility that over a longer observation period, subtle differences in FI and/or EE may have occurred. Indeed, there seemed to be a diverging pattern of cumulative FI between the two groups with a surprisingly lower cumulative FI in pTx animals (Fig. 2A), whereas absolute cumulative values either after 3 or 4 days did not reveal any statistical

difference (Table 1). It should be noted that number of animals decreased per day since, initially, mice were not tested for four days (see also methods). However, it seems rather unlikely that small changes in FI or EE are at all detectable due to large inter-individual and inter-litter variations. FI for example varied between 1.5 and 3.7 g during day 1 (i.e. when animals were more stressed due to placement from adaptation into measurement cages) and between 2.5 and 4.8 g during subsequent days 2-4 when FI was stable for individual mice.

It was previously reported that transplantation of eWAT to subcutaneous location reduced endogenous WAT mass without gross changes in body composition, body weight or FI (8; 9). In contrast, we demonstrated herein that transplantation of portal vein-drained adipose tissue increased endogenous WAT mass without affecting body weight or FI. Such findings points towards a crucial role for drainage site of adipose transplants on whole body fat mass regulation: transplants in the studies by Rooks et al. (8) and Konrad et al. (9) were drained systemically and resulted in reduced endogenous fat mass whereas transplants reported herein were drained into the portal vein resulting in increased endogenous WAT mass. These data may suggest that endogenous fat tissue may have the ability to control total body fat mass. Such control mechanism appears to be dependent on venous drainage of fat tissue, as it remains intact in mice receiving a systemically drained, but not in mice receiving a portally drained fat transplant. It is conceivable that factors released by adipose tissue (or the fat transplant) are extracted by their first liver passage and thereby their effect on fat mass control is lost.

Of note, in our previous study (12), we did not observe a major influence of portal vein-drained adipose tissue transplants on endogenous WAT mass. In contrast to the study presented herein, mass of transplanted WAT was approximately half of the amount used in the current study (~150 mg vs. ~280 mg) suggesting a dose-dependence and/or a “threshold” amount of transplanted fat tissue necessary to cause feedback mechanisms involved in sensing WAT mass. In contrast, development of glucose intolerance was not influenced by the amount of WAT mass transplanted since glucose tolerance was similarly impaired in the previous compared to the current study (data not shown). The notion of a certain “threshold” for sensing increased WAT mass is supported by a study from Foster et al. (26). In the latter study, 100-150 mg of either mWAT or eWAT was transplanted to the

intestine (i.e. to a portal vein-drained location) of mice resulting in a small but non-significant increase in endogenous fat mass.

Potentially, physical attachment of the two fat pads to the intestine may have affected intestinal motility or food absorption thereby impacting on FI either via modulation of intestinal vagal afferent signals to the brain or via inhibition of liver lipid oxidation (11). Our metabolic data, however, do not support such notion since neither food intake nor energy expenditure were different between pTx and sham-operated mice. Nevertheless, occurrence of nutrient mal-absorption and subsequent alterations of central feedback mechanisms cannot be ruled out in pTx mice since fecal composition and energetic value were not determined. Likewise, loss of innervation and/or re-innervation of transplanted adipose tissue may have affected endogenous fat mass since WAT innervation was proposed to sense WAT mass and contribute to its regulation (27-30).

Protein levels of PPARγ, which is considered as master regulator of adipogenesis (31; 32), were not altered to great extent in endogenous eWAT of both pTx and sham-operated control mice. Likewise, protein concentrations of upstream transcription factors such as CEBPβ and CEBPα were not changed either. Similarly, Srebf1 and SREBP-1c levels were not different between sham-operated and pTx mice suggesting that other mechanisms are primarily involved. However, this does not rule out the possibility that alterations in posttranslational modification/degradation of PPARγ such as ubiquitination or SUMOylation or that stress kinases like extracellular signal-regulated kinase (ERK) or p38 mitogen-actived protein kinase (MAPK) may influence PPARγ phosphorylation and hence impact on its activity level. An alteration of PPARγ or its activity would suggest an increase in WAT mass via hyperplastic response. We did not measure adipocyte size and number herein so far, but we previously did not observe any differences in fat cell size of eWAT and mWAT as evaluated from histological sections between pTx and sham-operated mice (12). However, a hypertrophic response cannot be ruled out and needs to be evaluated in future. Assuming that there was no difference in adipocyte size in the present study the increase in adipose tissue mass would suggest an increase in adipocyte number and, thus, rather a hyperplastic response, possibly downstream of PPARγ. Hence targets like lipoprotein lipase, fatty-acid transport protein, phosphoenolpyruvate carboxykinase or glycerol kinase need to be assessed to support such notion (32).

In summary, results presented herein demonstrate an increase in endogenous fat mass in mice receiving a portal vein-drained adipose tissue transplant. Accumulating evidence suggest that endogenous fat tissue may have the ability to control total body fat mass. However, such control mechanism may appear to be dependent on venous drainage of fat tissue, as it remains intact in mice receiving a systemically drained, but not in mice receiving a portally drained fat transplant. Due to the complexity of the model and the rather small changes in WAT mass in chow-fed animals, involved mechanisms may be difficult to identify. It is conceivable that factors released by adipose tissue (or the fat transplant) are extracted by their first liver passage and thereby their effect on food intake/energy expenditure is lost. Alternatively, neural feedback loops involving liver, brain and white adipose tissue that are involved in sensing an increase in adipose tissue mass may be disturbed in pTx mice.

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Figure Legends

Fig. 1 Correlation of body mass at sacrifice with adipose tissue mass of dissected depots for all animals (n = 41) analyzed (A) and split-up by groups for sham (B, n = 19) and portal-transplanted (C, n = 22) animals is depicted. Weights of different fat depots in sham (●) and portal-transplanted (▲) animals (D). Data are scatter plots with mean + SD for n = 19-22 animals. P-values of Student's t test or Mann-Whitney-U test are indicated above each fat depot.

Fig. 2 Cumulative food intake (g/mouse) is indicated over a period of 96 hours. Of note, number of animals decreases from day 1 (n=10 sham vs. 12 pTx) to day 4 (n=5 sham vs. 7 pTx) (A). Time course analysis for energy expenditure (cal/h) for 96 hours is indicated (B). Total ambulatory activity in a 2-D grid (total beam interruptions/h) is indicated for a period of 96 hours (C).

Fig. 3 Semi-quantitative gene expression analysis of uncoupling-protein 1 (Ucp-1) and Cidea in inguinal white adipose tissue (iWAT) (A), fibroblast growth factor 21 (Fgf21) in liver (B) and sterol regulatory element-binding factor 1 (Srebf1) as well as insulin-induced gene 1 (Insig1) in epididymal white adipose tissue (eWAT) is shown (C) for sham (●) and pTx (▲) animals. Data are scatter plots with mean + SD of n = 7 animals per group.

Fig. 4 Protein levels in epididymal white adipose tissue (eWAT) for indicated proteins or phosphorylation state of indicated proteins is shown. Semi-quantitative data normalized to the expression level of actin are indicated below for sham (black bars) and pTx (open bars) animals and represent mean + SD of n = 7 animals per group.

Figure 1

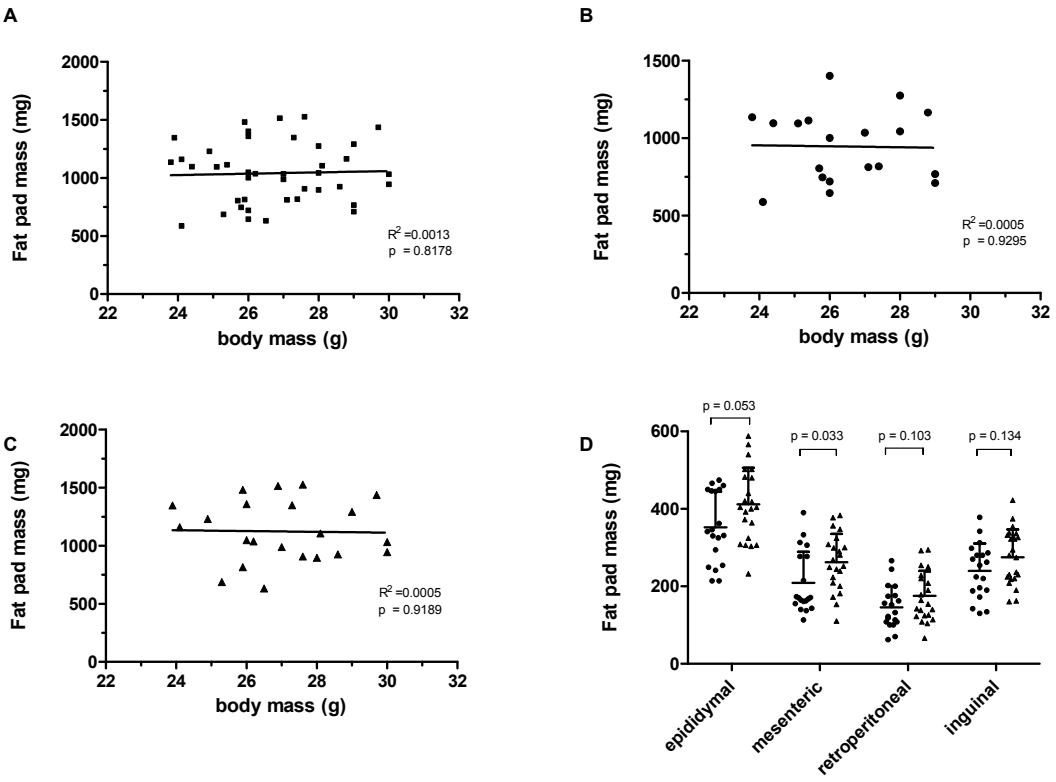

Figure 2

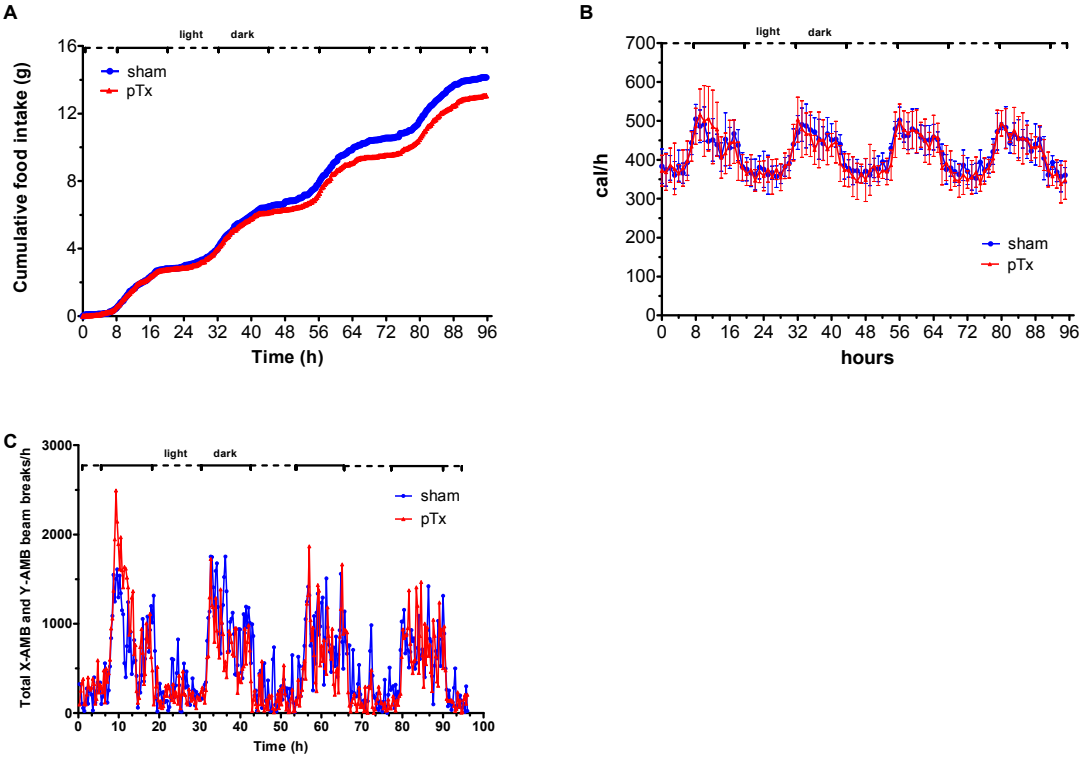

Figure 3

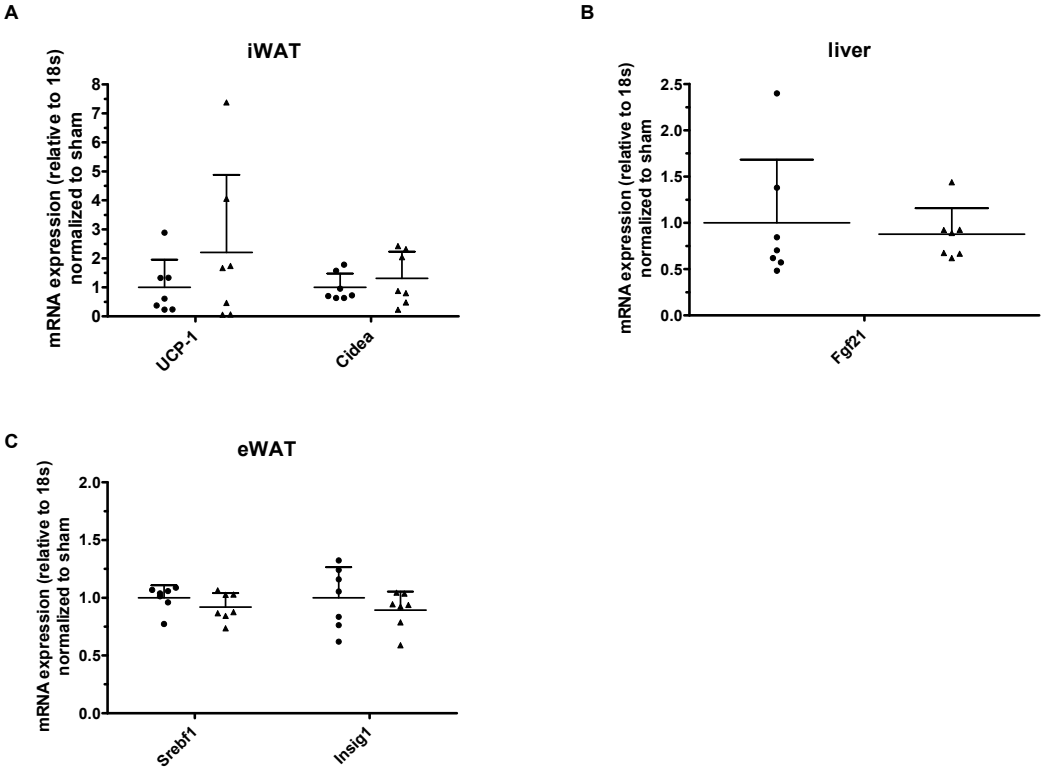

Figure 4

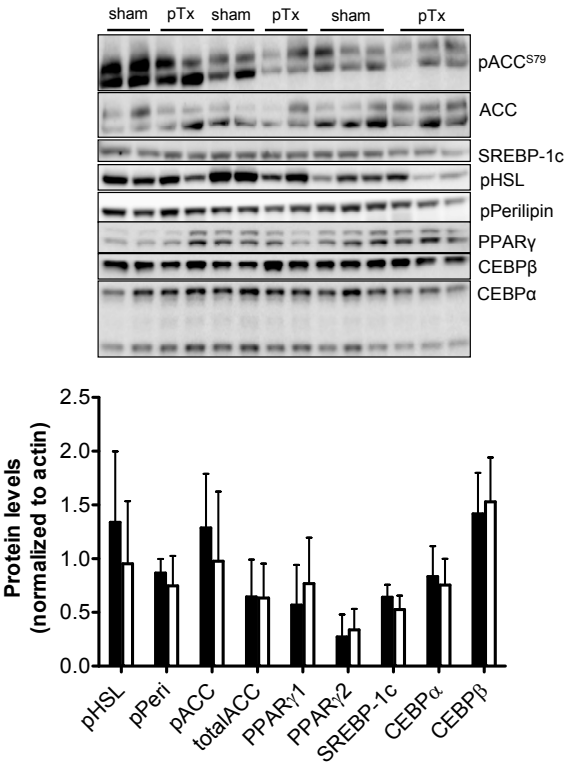

Table 1. Phenotypic characteristics of sham and pTx mice

	sham	pTx	p-value	
body mass pre surgery (g)	25.5 ± 2.3	26.4 ± 2.5	0.248	
body mass at sacrifize (g)	26.5 ± 1.6	27.0 ± 1.8	0.289	
Δ body mass (g)	0.9 ± 1.7	0.6 ± 1.3	0.513	
total fat pad mass (mg)	946.2 ± 229.2	1123.8 ± 265.2	**0.029**	
Food intake (g/mouse) day1	2.9 ± 0.5	2.9 ± 0.6	0.906	
Food intake (g/mouse) day2	3.6 ± 0.7	3.5 ± 0.6	0.634	
cumulative food intake day1-day4 (g/mouse)	14.1 ± 2.1	13.1 ± 2.0	0.39	
cumulative food intake day2-day4 (g/mouse)	11.0 ± 1.9	10.2 ± 1.4	0.387	
EE (cal/mouse) day1	9967 ± 374	9990 ± 836	0.79	unadj.
EE (cal/mouse) day1	10083 ± 384	9897 ± 513	0.435	adj.
EE (cal/mouse) day2	10006 ± 238	9794 ± 818	0.553	unadj.
EE (cal/mouse) day2	10073 ± 240	9744 ± 672	0.306	adj.
activity (breaks/day/mouse) day1	42270 ± 21750	50591 ± 35990	0.574	
activity (breaks/day/mouse) day2	50856 ± 12148	36635 ± 19163	0.139	
average RQ (24h) day1	0.91 ± 0.08	0.90 ± 0.08	0.141	
average RQ (24h) day2	0.95 ± 0.07	0.95 ± 0.07	0.668	

Results are mean ± SD of 7-22 animals dependent on parameter measured. Δ = delta, EE = energy expenditure, RQ = respiratory quotient. Statistical values are indicated. adj. = adjusted, unadj. = unadjusted

Table 2. Fold difference in expression of microarray transcripts regulated in eWAT between sham (control) and pTx (treatment) mice

Acession	Name	Fold change	P-value
NR_037297	Mir3110	-1.48	0.000434
NM_009721	Atp1b1	-1.64	0.001022
NM_011134	Pon1	1.51	0.002779
NR_029575	Mir24-1	-1.50	0.01827
NR_039584	Mir5123	-2.28	0.01901
NM_146986	Olfr38	1.43	0.02076
NM_008682	Nedd1	1.60	0.02124
NR_003519	Pisd-ps2	1.43	0.02264
NM_008030	Fmo3	-1.80	0.02736
NR_029580	Mir194-1	-1.42	0.02935
ENSMUST00000056571	Syne1	1.47	0.03172
NM_001146275	Iigp1	1.47	0.03569
ENSMUST00000023151	Bcl6	-1.74	0.04839
ENSMUST00000003100	Cyp2f2	1.42	0.0726
NM_018866	Cxcl13	-1.74	0.08465
NR_037276	Mir3093	1.44	0.09061
ENSMUST00000128411	Tgtp2	1.49	0.09744

First accession code of raw data is given, common gene name, fold change to control and p-value. Data are sorted by p-value.

Table 3. Fold difference in expression of microarray transcripts regulated in mWAT between sham (control) and pTx (treatment) mice

Accession	Name	Fold change	P-value	Accession	Name	Fold change	P-value
NR_003517	Pisd-ps1	-1.79	0.0009835	NM_001008533	Adora1	1.73	0.04463
NR_003518	Pisd-ps3	-1.47	0.01382	NM_153170	Slc36a2	1.50	0.04623
ENSMUST00000056571	Syne1	1.91	0.001053	NM_013739	Dok3	-1.62	0.04652
NR_027656	Oaz1-ps	-1.59	0.001156	NM_009098	Rps8	-1.46	0.04654
NM_152809	Csnk1g3	-1.60	0.001917	NM_001170537	Mef2c	-1.44	0.04663
ENSMUST00000002678	Tgfb1	-1.46	0.002325	NM_00115996	Ralgps2	-1.61	0.04666
NR_039580	Mir5120	-2.21	0.002526	NM_001038018	Grk6	-1.49	0.04857
NM_177204	Fam40b	1.67	0.002621	NM_016803	Chst3	-1.48	0.04941
NM_021362	Pappa	1.45	0.003055	NM_001146022	Wdfy4	-1.48	0.05071
NM_030207	Sfi1	-1.45	0.003277	NM_011600	Tle4	-1.42	0.05213
NM_016758	Rgs14	-1.42	0.003409	NM_007641	Ms4a1	-1.64	0.05278
NM_001039493	Plekhm3	-1.47	0.003505	NM_182806	Gpr18	-1.53	0.05316
NM_178652	Supt3h	1.46	0.004494	NM_001145858	Sh3bp2	-1.50	0.05365
NM_013470	Anxa3	1.47	0.005721	NM_026184	Ero1lb	-1.57	0.05415
NM_183319	Xkrx	-1.49	0.006361	NM_001077353	Gsta3	1.70	0.05484
NM_025983	Atp5e	1.44	0.00679	ENSMUST00000033063	Cd37	-1.47	0.05488
ENSMUST00000005798	Snx6	1.43	0.006836	NM_010742	Ly6d	-1.57	0.05563
NM_009022	Aldh1a2	1.90	0.007158	NM_010288	Gja1	1.64	0.05596
NM_001013817	Sp140	-1.80	0.007207	NM_013545	Ptpn6	-1.47	0.05655
NM_144945	Lgi2	1.52	0.008759	NM_025730	Lrrk2	-1.46	0.05762
NM_011076	Abcb1a	1.57	0.009086	NM_024444	Cyp4f18	-1.49	0.05831
NM_153194	Zfp740	-1.44	0.009198	ENSMUST00000102582	Acacb	1.61	0.05849
NM_007489	Arntl	-1.41	0.009407	NM_028089	Cyp2c55	1.64	0.05917
NM_010436	H2afx	-1.72	0.009418	NM_145835	Lctl	2.12	0.05968
NM_007799	Ctse	-1.44	0.009698	NM_009844	Cd19	-1.66	0.05972
NM_001163288	Susd1	-1.45	0.01052	ENSMUST00000026148	Cbr2	1.58	0.06022
NM_010877	Ncf2	-1.45	0.01109	NM_139138	Emr4	-1.42	0.06083
NM_008329	Ifi204	1.50	0.01121	ENSMUST00000038829	Faim3	-1.51	0.06099
NM_134122	Nrm	-1.52	0.01294	NM_019521	Gas6	1.63	0.06119
NM_008207	H2-T24	-1.63	0.01358	ENSMUST00000007317	Krt19	1.42	0.0612
NM_010191	Fdft1	1.53	0.01451	NM_172930	Fam70a	1.85	0.06305
NR_035428	Mir1196	-1.70	0.01502	NM_008690	Nfkbie	-1.42	0.06498
NM_001163555	Pou2f2	-1.57	0.01512	NM_026728	Echdc2	1.45	0.06499
NM_025895	Med28	1.49	0.01533	ENSMUST00000106847	Trim34b	-1.42	0.06571
ENSMUST00000022124	Cd180	-1.47	0.01637	NM_025325	Haao	-1.59	0.06735
ENSMUST00000003469	Cd79a	-1.46	0.01669	NM_130449	Colec12	1.47	0.06769
NM_025429	Serpinb1a	-1.48	0.01818	NM_025855	Echdc1	1.52	0.06828
ENSMUST00000119129	Cdon	1.70	0.01879	NM_172778	Maob	1.55	0.06924
NM_001163336	Atp2a3	-1.56	0.02013	NM_013517	Fcer2a	-1.57	0.0709
NM_009924	Cnr2	-1.48	0.02016	NM_008528	Blnk	-1.62	0.07098
ENSMUST00000108430	Rps19	-1.81	0.02101	NR_001579	Terc	-1.52	0.07177
ENSMUST00000108430	Rps19	-1.81	0.02101	NM_001099328	Zfp831	-1.49	0.07187
NR_028532	Snord71	-1.48	0.02174	NM_001033350	Bank1	-1.52	0.07238
ENSMUST00000171445	Eps8l1	-1.52	0.02195	NM_009302	Swap70	-1.44	0.07247
ENSMUST00000085573	Traf5	-1.46	0.02432	NM_020044	Lat2	-1.42	0.07272
NM_178759	Timd4	-1.66	0.02457	ENSMUST00000014174	Pax5	-1.44	0.07318
NM_017370	Hp	2.57	0.02547	NM_010566	Inpp5d	-1.42	0.07505
NM_001081066	Dennd3	-1.49	0.0262	ENSMUST00000031171	Stap1	-1.47	0.07733
NM_001162938	Pydc3	-1.64	0.02626	NM_008279	Map4k1	-1.42	0.07898
NM_010678	Aff3	-1.46	0.02704	NM_008206	H2-Oa	-1.42	0.07902
NM_027127	Gpx8	1.55	0.02791	NM_024499	Sgta	1.45	0.07947
NM_177353	Slc9a7	-1.75	0.02793	NM_009835	Ccr6	-1.58	0.07968
NM_172285	Plcg2	-1.54	0.02839	NM_028010	Apol6	1.52	0.08001
NM_011607	Tnc	-1.66	0.02854	NM_025863	Trim59	-1.48	0.08153
NM_00116250	Parvg	-1.48	0.02898	NM_199012	Fchsd2	-1.45	0.08325
NM_010164	Eya1	-1.47	0.03011	NM_001042711	Amy2a5	3.02	0.08372
NM_001043317	Cd22	-1.68	0.03128	ENSMUST00000171223	Fgr	-1.49	0.08388
NM_019866	Spib	-1.96	0.03148	NM_183222	Fcrl5	-1.51	0.08403
NM_178087	Pml	-1.46	0.0327	NM_146042	Rnf144b	1.42	0.08468
NM_001177438	Aldh3b2	1.70	0.03466	NM_013602	Mt1	1.46	0.08522
NM_008630	Mt2	1.48	0.03529	ENSMUST00000059349	Tlr1	-1.43	0.08711
NM_013482	Btk	-1.56	0.03541	ENSMUST00000015889	Plekho1	-1.44	0.08819
ENSMUST00000040250	Kmo	-1.68	0.03599	NM_026172	Decr1	1.52	0.08841
NM_025848	Sdhd	1.45	0.03697	NM_145839	Rasgef1b	-1.47	0.08878
NR_039563	Mir5104	-1.55	0.03753	NM_016707	Bcl11a	-1.53	0.0897
ENSMUST00000059244	Olfr820	-1.56	0.03799	NM_007551	Cxcr5	-1.42	0.09081
NM_199366	Gal3st2	-1.53	0.03869	NM_011426	Siglec1	-1.43	0.09139
ENSMUST00000089860	Fam13a	1.58	0.03928	NM_031842	Smarcd1	1.62	0.09149
NM_00125205	Ly6c1	1.52	0.03988	NM_030026	Mccc2	1.44	0.09421
NM_19922	Cd300c	-1.42	0.04007	NM_153090	Fcrl1	-1.60	0.09463
ENSMUST00000027409	Des	-1.44	0.04013	NM_134042	Aldh6a1	1.44	0.09552
NM_178712	Gpr64	1.49	0.04035	NM_178924	Upk1b	1.94	0.09602
NM_007549	Blk	-1.88	0.04145	NM_028075	Tnfrsf13c	-1.54	0.09808
NM_019549	Plek	-1.57	0.04203	ENSMUST00000073080	Cycs	1.46	0.09845
NM_183175	C1qtnf9	1.49	0.04209	NM_011313	S100a6	1.57	0.099
NM_133895	Slc15a4	-1.48	0.04213	NM_001111059	Cd34	1.43	0.09982
NM_201518	Flrt2	1.65	0.04363				

First accession code of raw data is given, common gene name, fold change to control and p-value. Data are sorted by p-value.

6. General discussion

During the time course of performing the experiments for this thesis starting in 2010, several other studies have been published (54-56; 210-215) concerning the influence of a short bout of HFD-feeding on the development of IR and other important parameters, which may respond instantaneously on this dietary challenge, partly supporting and contradicting the results presented herein. Subsequently, our findings will be compared to these studies and further implications will be discussed.

Studies performed with short time periods of challenging mice or rats with HFD in the range of 2-7 days showed smaller or larger contributions of the innate immune system to the development of IR at this early stage. Apparently, mouse-strain dependent reaction in general on HFD feeding (216) and possibly also on the short-term overfeeding does play a role even in the commonly used C57BL/6 strain as publications do not clearly indicate if there is e.g. WAT expansion at such early phase and if, together with its underlying functional changes, this contributes as a single factor to the development of IR in susceptible animals (217). This, together with the inherent properties of WAT depots (86) as well as reactivity and predictability of gene alterations in a HFD-setting (215), and tissue specificity in reaction proven in long-term DIO mice for WAT, muscle, liver, and heart (218), may contribute to the differences in findings between papers published.

In general, it turns out that liver is the primary and first organ to develop insulin resistance as early as 3-4 days after initiating HFD feeding (56; 214; 219), which is in accordance with our data. Interestingly, two studies (56; 214) did not find an aggravation of whole body glucose intolerance as assessed by a glucose tolerance test after 3 days of HFD-feeding. However, these findings do not exclude that different mechanisms causing this state of insulin resistance are involved at later stages of HFD-induced IR.

Subsequently, after 7 days of HFD-feeding, WAT may become insulin resistant as assessed by hyperinsulinaemic-euglycaemic clamp technique, and, similarly as liver, its insulin resistance was not further aggravated during continuous HFD feeding (214). At least in a shorter setting, i.e. 3-4 days, WAT seems to remain fully functional as could be shown in our study and more recently in another publication (54). However, a further study suggested impaired WAT lipolytic activity regulated by central mechanism due to the development of brain insulin resistance

(213). Apparently, discerning central and peripheral insulin action in this short-term HFD setting at the same time could resolve these paradox findings. The functionality of WAT seems to persist despite showing a certain degree of inflammation as assessed by gene expression levels of pro-inflammatory cytokines in several studies together with adipose tissue mass expansion (54-56). The latter effect may occur dependent (54-56) or independent (214) of total body mass changes or do not occur at all as was demonstrated in our animals. Again, it is pretty likely that the effect of increased body weight gain during this short period of HFD-feeding and the dependent or independent effect of WAT mass increase may not be causally involved in the development of glucose intolerance or insulin resistance, at least nicely shown for the development of central insulin resistance (210) but that rather inherent production and release of cytokines (54), a switch in dynamic immune response as evidenced by activation of adipose-resident NKT cells (55) or transient neutrophil infiltration (220) play a major role at this early stage.

Similarly, lipid overload in tissues and circulation either by acute infusion of lipids (221; 222) or in these short-term HFD settings (54-56), which are known to lead to a transient hyperphagic response (223; 224), altering circadian rhythm (212) and stimulate anorexigenic mechanisms (211), is also suggested to be causally linked to the development of both hepatic and total body insulin resistance. Interestingly, other theories propose that the development of hepatic insulin resistance is rather a defence mechanism against further aggravation of disease development (225). Apparently, lipid overload may either cause or contribute to the development of insulin resistance at various degrees in different tissues. Turner et al. (214) observed liver TAG and DAG to be elevated after one week of HFD-feeding without alterations of ceramide levels, which are known to be able to directly interfere with insulin signalling. WAT may be otherwise affected as neither TAG nor DAG but rather number of ceramides and sphingomyelins were elevated. Furthermore, skeletal muscle lipid content was not altered until skeletal muscle insulin resistance developed after three weeks of HFD-feeding in their study. Furthermore, the possibility exists that there are also time-dependent compensations or alterations of liver lipid content, which can contribute to the degrees of hepatic insulin resistance (226). It is likely that this may explain the shift towards higher lipid contents in skeletal muscle and WAT at later stages observed by Turner et al. (214).

This strongly suggests different roles of tissue lipid overload in the three main insulin-responsive tissues and hence our data, which discern development of hepatic steatosis from inflammation, demonstrate further the complexity of correctly pinpointing *the* underlying reason in the development of insulin resistance.

Hence, to date it remains still impossible to exactly trigger the initial mechanism leading to the development of hepatic insulin resistance at the very beginning of an obesogenic HFD-challenge. Possibly WAT inflammation and lipid accumulation in target tissues play a concomitant role in exaggerating each other's single effect on insulin signalling and inter-organ cross talk is continuously involved in this process (69).

Similarly, transplanting WAT has gained attention in different contexts by several studies (227-233). The study of Foster et al. (227) confirmed previous findings that eWAT removal is a powerful signal for other fat depots to increase endogenous mass, something that has been known in rodents for decades (234). Their findings also suggested that for true visceral adipose tissue, i.e. mWAT, less fat mass needs to be removed when compared to eWAT to exert positive effects on glucose metabolism. However, their study did not demonstrate any negative regulatory effects of portal transplanted mWAT on fat mass. This may have been due to the small mass of transplanted tissue, which may be below a certain threshold necessary to exert regulatory responses. Similarly, the same group performed a series of different donor tissue transplantations into the visceral cavity by attaching these autologous fat pads to endogenous mesenteric adipose tissue using tissue glue (228). Hence, it is likely that these fat pads may exert their effect also via the endogenous mWAT as direct WAT-WAT interaction may have occurred (235; 236). Despite reporting a positive effect of transplanting iWAT to the mesentery on hepatic glucose and lipid metabolism, and only a statistical measurable increase in rWAT when mWAT was transplanted and eWAT when iWAT was transplanted, the authors did not observe any other alterations of endogenous WAT mass. This again may have been due to the low amount of transplanted fat (~100 to 150mg), much less than was used in the current study (~280mg). Nevertheless, these studies demonstrate that transplantation of different WAT depots into the visceral cavity draining into the portal vein exerts alterations on endogenous WAT depots and functions (234; 237).

WAT transplantation has been recently suggested to protect in part from spontaneously developing T1DM in BB/OK rats (229). In this study, the authors took eWAT and transplanted it into subcutaneous sites, which was previously shown to exert positive effects on whole body metabolism (65). Similarly, Gunawardana et al. (238) found that mice were protected from developing streptozotocin-induced T1DM after transplanting BAT into subcutaneous locations. In both studies, normalizing WAT function seemed to be a major mechanism for protecting against T1DM. However, none of the studies assessed transplantation-induced endogenous WAT mass alterations in these T1DM models.

Barrera et al. (230) used an interesting approach of injecting eWAT of 12-week HFD-fed animals into recipient mice either into subcutaneous area or directly into recipient eWAT. At least the latter approach may have led to increases in the endogenous eWAT depot additional to having intact sympathetic nervous innervation - a suggested sensor of WAT mass. Of note, eWAT injection lead to deterioration of glucose and insulin tolerance as well as increased body weight gain suggesting that HFD-fed donor tissue triggers powerful signals when transplanted into the “wrong” side as subcutaneously grafted animals remained similar to control animals. However, these authors also did not test for endogenous WAT mass alterations which would have been a valuable point in discerning “unhealthy”, i.e. diet-induced obese WAT, function from transplant location.

A study similar to ours (231) found increased portal IL-1β to be not only a regulator of hepatic gluconeogenic flux but also critical for suppressing WAT mass expansion as IL-1β knockout animals demonstrated higher eWAT mass in both chow-fed and HFD-fed conditions. We have not tested IL-1β’s role in our animals so far.

Lastly, the study of Zhao et al. (232) points to a crucial role of neovascularisation of fat grafts for their function and survival. Although their study does not report completely new findings (239), it reminds us that neovascularisation/revascularisation of fat transplant should be more frequently assessed in further research studies to be able to better compare outcomes of different fat grafts, their inherent characteristics, and their transplantation site-dependent effects on metabolism.

Collectively, the projects of this PhD thesis add to the body of evidence that WAT exerts a myriad of functions and contributes greatly to the development of obesity-associated insulin resistance. Beyond that, it has powerful auto-regulatory mechanism to control its own mass and this ability may be in part dependent on its drainage site.

7. References

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